RESULTS

Correlation between serum IgE levels and IFN-γ production by PHA-stimulated and IL-18–stimulated PBMCs

The IL-18–stimulated PBMCs of the atopic group (n = 39; mean ± 1 SD = 161.1 ± 308.2 pg/mL) had lower IFN-γ production than those of the control group (n = 41; mean ± 1 SD = 429.0 ± 728.7 pg/mL; *P* = .0370). We investigated the correlation between serum IgE levels and concentrations of IFN-γ in the atopic and control subjects produced by IL-18–stimulated PBMCs. The serum IgE levels were negatively correlated with the concentrations of IFN-γ produced by IL-18–stimulated PBMCs (*P* = .0359; *r* = 0.235; Fig 1). The concentrations of IFN-γ in the atopic and control subjects produced by IL-18–stimulated PBMCs were well correlated with those produced by PHA–stimulated PBMCs (*P* = .00370; *r* = 0.432). The concentrations in the atopic and control subjects of IFN-γ produced by IL-12–stimulated PBMCs were significantly correlated with those produced by IL-18–stimulated PBMCs (*P* = .00529; *r* = 0.490). However, in several patients, IFN-γ was not sufficiently produced by IL-18 stimulation, though IFN-γ was sufficiently produced by PHA or IL-12 stimulation. We used a multiple regression model to simultaneously evaluate the effects of the log-transformed independent variables on IFN-γ production and incorporate the effect of IL-12, IL-18, or PHA as a categorical grouping variable. It showed no significant correlation between serum IgE level and IL-12–stimulated, IL-18–stimulated, or PHA-stimulated IFN-γ.

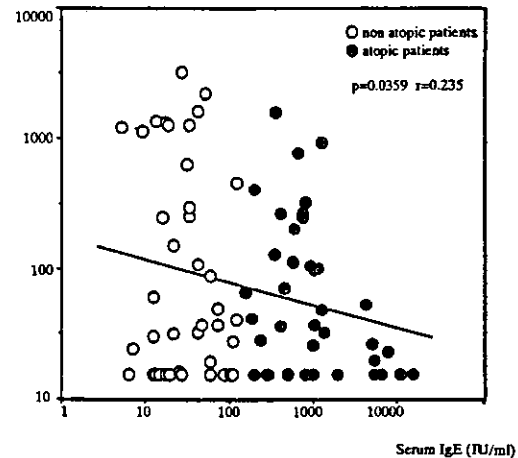


FIG 1. The relationship between serum IgE levels and IFN-γ concentrations in the cultured supernatants of IL-18–stimulated PBMCs. The serum IgE levels were negatively correlated with the IFN-γ concentrations by IL-18–stimulated PBMCs. (*r* = 0.235; *P* = .0359). *Open circles* represent nonatopic subjects; *closed circles* represent atopic subjects. The line is a recurrence straight line.

Three-base deletion in IL-18R α chain transcript

To clarify the mechanism of selectively reduced IFN-γ production in response to stimulation with IL-18 and high serum IgE level, IL-18R α chain cDNAs from atopic patients were sequenced. We identified a 3-base deletion of the IL-18R α chain cDNA (*950delCAG*) in some patients. By this 3-base deletion, the codon for Ala 317 was deleted. Because the transmembrane domain of IL-18R α spans Gly 330 and Tyr 351, this Ala was located in the extracellular domain. We cloned the partial genomic DNA around *950delCAG* by PCR. This deletion was not detected in the genomic DNA. The genomic sequence suggested that the *950delCAG* mutation was caused by alternative splicing (Fig 2, *A*).

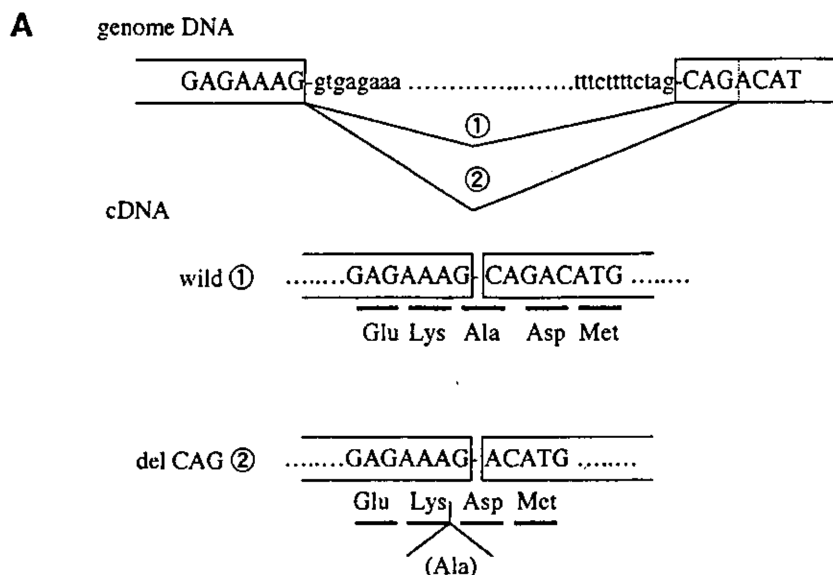
We investigated the expression pattern of *950delCAG* of IL-18R α chain transcript in PBMCs. Three patterns were identified by polyacrylamide gel electrophoresis: predominant expression of the wild-type transcript (wild/wild; Fig 2, *B*, *lane 5*), almost equal expression levels of the 2 transcripts (wild/delCAG; Fig 2, *B*, *lane 3*), and predominant expression of *950delCAG* transcript (delCAG/delCAG; Fig 2, *B*, *lane 4*). We investigated the frequency of plasmid DNA with wild or *950delCAG* from randomly selected colonies; this is derived from the PCR product of each PBMC's cDNA (Table I). Consistent with the results of the polyacrylamide gel electrophoresis, the colonies originating from wild/wild had 6/6 to 8/8 wild-type cDNA. The colonies originating from delCAG/delCAG had 7/7 to 10/10 *950delCAG* cDNA. The colonies originating from wild/delCAG had 50–66.7% wild DNA and 50–33.3% *950delCAG* DNA.

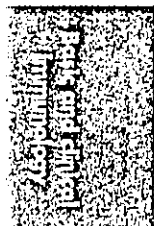
Expression pattern of *950delCAG* IL-18R α chain transcript in the atopic patients

We investigated whether the expression pattern of the *950delCAG* is associated with atopic patients. The χ^2 test revealed a significant difference (*P* = .0179) in the expression pattern of *950delCAG* of IL-18R α chain transcript between the atopic group and the nonatopic group (Table II). We next investigated whether there was a correlation between the expression pattern of *950delCAG* of IL-18R α chain transcript and IL-18–induced IFN-γ production. Interestingly, the expression of delCAG/delCAG significantly reduced IFN-γ production in comparison with the expression of the wild/wild (*P* = .0080; Fig 3, *A*). In contrast, there was no significant difference between the expression pattern of *950delCAG* of IL-18R α chain transcript and IL-12–induced IFN-γ production (Fig 3, *B*). People who had the expression pattern of delCAG/delCAG transcripts had high serum IgE levels in comparison with people who had the wild/wild (*P* = .0209; Fig 4).

DISCUSSION

In the present study, we showed that the concentrations of IFN-γ produced by IL-18–stimulated PBMCs in the atopic group were significantly lower than those in





B

FIG 2. A, Wild and *950delCAG* of IL-18R α chain sequences of genomic DNA and cDNA. The *950delCAG* was generated by alternative splicing. **B,** Three expression patterns (wild/wild, wild/delCAG, and delCAG/delCAG) identified by polyacrylamide gel electrophoresis. *Lane 1:* The template DNA is plasmid DNA with *950delCAG* of IL-18R α chain. *Lane 2:* The template DNA is plasmid DNA with wild IL-18R α chain. *Lane 3:* The PCR product was identified as wild/delCAG. *Lane 4:* The PCR product was identified as delCAG/delCAG. *Lane 5:* The PCR product was identified as wild/wild.

TABLE I. Frequency of randomly selected colonies harboring wild or *950delCAG* of IL-18R α chain cDNA

	Wild colony no./total colonies no. (%)*	*950delCAG* colony no./total colonies no. (%)*
Wild/wild†		
Control 1	8/8 (100%)	0/8 (0%)
Control 2	8/8 (100%)	0/8 (0%)
Control 3	6/6 (100%)	0/6 (0%)
Wild/delCAG*		
Control 4	4/7 (57.1%)	3/7 (42.9%)
Allergy 1	4/6 (66.7%)	2/6 (33.3%)
Allergy 2	5/10 (50%)	5/10 (50%)
delCAG/delCAG*		
Allergy 3	0/7 (0%)	7/7 (100%)
Allergy 4	0/9 (0%)	9/9 (100%)
Allergy 5	0/10 (0%)	10/10 (100%)

*Determined by sequencing.
†Determined by polyacrylamide gel electrophoresis.

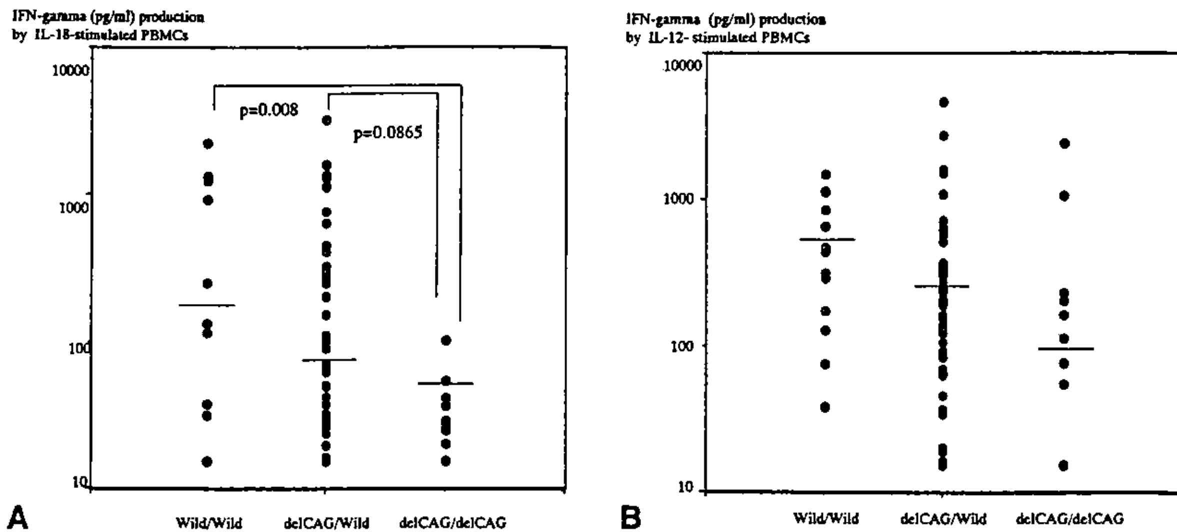


FIG 3. A, The relationship between expression pattern of *950delCAG* of IL-18R α chain transcript and IL-18–induced IFN-γ production. IFN-γ production was significantly lower when delCAG/delCAG was expressed than when wild/wild was expressed (P = .0080). *Bar* shows geometric mean. **B,** The relationship between the expression pattern of *950delCAG* IL-18R α chain transcript and IL-12–induced IFN-γ production. There was no correlation between the case when delCAG/delCAG was expressed and that when wild/wild was expressed or between the case when delCAG/delCAG was expressed and that when wild/delCAG was expressed. *Bar* shows geometric mean.

the control group. Moreover, serum IgE level was negatively correlated with the concentrations of IFN-γ produced by PBMCs cultured with IL-18. However, in several patients, IFN-γ production was not sufficiently induced by IL-18 stimulation, though IFN-γ production was sufficiently induced by IL-12 or PHA stimulation. These results suggest that there might be some abnormalities in signal transduction of IL-18. Therefore, we analyzed the IL-18R α chain cDNA sequence in these atopic patients. We identified a 3-base deletion of *950delCAG* of IL-18R α chain cDNA in some atopic patients. The *950delCAG* mutation resulted in the deletion of 317 Ala, which was located in the extracellular domain of IL-18R α. PBMCs from the patients with low IFN-γ production stimulated by IL-18 tended to express the delCAG/delCAG transcript. Furthermore, this group showed a high serum IgE level. These results suggest that PBMCs from the patients in whom the delCAG/delCAG transcript was expressed could not suppress IgE production because of reduced IFN-γ production by IL-18 stimulation. Reduced IFN-γ production by IL-18–stimulated PBMCs might be caused by the impairment of the IL-18R signaling due to the deletion of Ala in the extracellular domain of IL-18R α chain cDNA.

Despite the significance, the correlation between serum IgE and IFN-γ concentrations produced by PBMCs cultured with IL-18 was weak. These results suggest that IgE is regulated by multiple factors, including cytokines and some receptors. Even cytokines IL-4 and IL-13 have been reported as IgE regulators.[16] The involvement of multiple factors in IgE regulation might cause the weak association between IL-18–stimulated IFN-γ production and IgE.

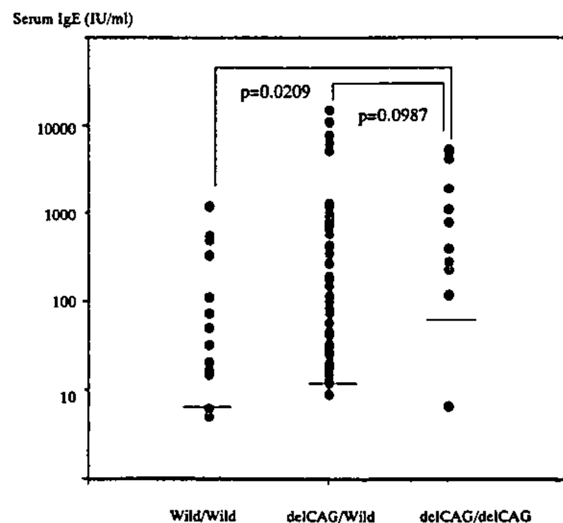


FIG 4. Relationship between the expression pattern of *950delCAG* of IL-18R α chain transcript and serum IgE levels. The serum IgE levels in the case when delCAG/delCAG was expressed were significantly higher than those when wild/wild was expressed (P = .0209). *Bar* shows geometric mean.

TABLE II. Relationship between the expression pattern of *950delCAG* of IL-18R α chain cDNA and allergy

	n	Wild/ wild*	delCAG/ wild*	delCAG/ delCAG*	*P* value
Control	41	9	30	2	
Atopy	39	4	26	9	P = .0179
Totals	80	13	56	11	

*Determined by polyacrylamide gel electrophoresis.

It is assumed that *950delCAG* might cause a loss of function of the IL-18R α chain. With regard to the loss of function due to *950delCAG*, there is the possibility that this mutation might result in a reduced binding affinity of the IL-18R α chain to IL-18 and might result in insufficiency of signal transmission. The levels of IFN-γ production by IL-18–stimulated PBMCs cultured for 3 days tended to be comparable to the normal level (data not shown). Therefore, PBMCs with *950delCAG* might require more time to produce IFN-γ at the same level as those of wild IL-18R α chain cDNA. This result suggests that the function of IL-18R α chain cDNA with the *950delCAG* results in the insufficiency of signal transduction.

Alternative splicing transcripts of cytokines and cytokine receptor genes have been reported in allergic patients. Airway biopsy specimens from asthmatic patients were more likely to express the IL-4 alternative splice variant IL-4δ2.[17] An altered expression and action of the low-affinity IgE receptor FcεRII (CD23) was found in the airway smooth muscle of asthmatic patients.[18] Deletion of individual exons and induction of soluble murine IL-5R α chain expression were also observed.[19] A predominant expression of alternative transcripts of cytokine and cytokine receptor genes might be one of the mechanisms of pathogenesis of atopic diseases.

It was originally considered that the major action of IL-18 was induction of IFN-γ production by T_H1 cells and NK cells, particularly in combination with IL-12.[20-24] In combination with IL-12, IL-18 also induces anti-CD40–activated B cells to produce IFN-γ, which inhibits IL-4–dependent IgE and IgG1 production.[25] However, the functions of IL-18 in vivo are very heterogeneous and complicated. Recently, it has been reported that IL-18 enhances the IL-12–driven T_H1 immune responses as well as stimulating T_H2 immune responses in the absence of IL-12.[26] It has been reported that IL-18 enhanced eosinophil recruitment into the airways[27] and, in combination with IL-2, increased IL-13 secretion by NK cells and T cells.[28] It has also been reported that in the presence of IL-3, IL-18 induced basophils and mast cells to express high levels of IL-4, IL-13, and histamine.[29] Recently, IL-18 was found to increase IgE production in a $CD4^+$ T-cell–dependent, IL-4–dependent, and STAT6-dependent manner.[30]

We have shown that when IL-18 alone was added to PBMCs, IL-18 induced T_H1-type cytokines such as IFN-γ and that IL-18 played an important role in the inhibition of atopic diseases. This is the first report showing that some atopic diseases are caused by impairment of the IL-18 signal cascade, which downregulates IgE production. In most cases, atopic disease is caused by multiple factors. In this report, we showed that 9 of 39 atopic patients expressed *950delCAG* and reduced IFN-γ production. We think that in these 9 patients dominant expression of *950delCAG* is one of the factors that caused elevated IgE. However, the other factors, such as IL-4 receptor and IL-13 polymorphism, might be involved in IgE elevation in these patients. The patients without dominant *950delCAG*, who showed the reduced IFN-γ production stimulated with IL-18, might have other polymorphisms, such as IL-18 or IL-18R β. We have not as yet determined what mechanism induces the predominant expression of the IL-18R α chain cDNA in some atopic patients. Cytokines, viral infections, and environmental factors might be the candidate modulators of IL-18R α chain transcript expression. Experiments along these lines are now under way.

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The correlation between ovomucoid-derived peptides, human leucocyte antigen class II molecules and T cell receptor-complementarity determining region 3 compositions in patients with egg-white allergy

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Summary

Background Food allergies are more prevalent in children, due to the immature gastrointestinal epithelial membrane barrier allowing more proteins through the barrier and into circulation. Ovomucoid (OM) is one of the major allergens that is found in egg white.

Objective The aim of this study was to determine T cell epitopes, antigen-presenting human leucocyte antigen (HLA) class II molecules of the T cell lines (TCLs) and T cell clones (TCCs), and complementarity determining region (CDR) 3 loops of the T cell receptor (TCR) α and β chains of the TCCs specific to OM.

Methods We established TCLs and TCCs specific to OM from peripheral blood mononuclear cells (PBMCs) of four atopic patients with egg-white allergy using a mixture of a panel of overlapping synthetic peptides corresponding to the amino acid sequence of the entire OM. We identified the T cell epitopes by antigen-induced proliferative responses, antigen-presenting molecules using allogeneic PBMCs and CDR3 loops of the TCR α and β chains by cloning and sequence analysis.

Results The TCLs and TCCs responded to seven different peptides, and their antigen-presenting molecules were different from each other. Sequence analysis of the TCR α and β gene usage of the TCCs showed marked heterogeneity, and the usage of the CDR3 loop of the TCCs involved heterogenous amino acid residues. Interestingly, TCCs 'IH3.3' and 'YT6.1' recognized the same OM peptides, and had the same TCR Vβ-Jβ gene usage. Considering that peptide motifs bind to HLA class II molecules, the electrically charged residue (positive or negative) on the CDR3α and the CDR3β loops of TCR of TCC may form ionic bonds with a charged residue on the HLA class II molecules-peptide complex.

Conclusions TCCs that have the same TCR gene usage were established from patients who had shown similar hypersensitivity-type, indicating that antigen recognition by a specific TCR is closely associated with the characteristics of each patient's symptoms.

Keywords complementarity determining region 3, ovomucoid, T cell clone, T cell epitope, T cell receptor

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Introduction

Food allergy has been demonstrated to play an important role in the pathogenesis of atopic dermatitis (AD), affecting approximately one-third of the children with AD [1]. It is also known that food allergies are more prevalent in children, due to the immature gastrointestinal epithelial membrane barrier allowing more proteins through the barrier and into circulation [2]. Among various food antigens, hen's egg, particularly its egg white, is one of the most common causes of food allergy in young children. Ovomucoid (OM), which constitutes 11 weight percentage of the total protein in egg white, is a highly soluble, heat-stable glycoprotein with a molecular weight of 28 000 [3, 4]. Chicken OM consists of three tandem homologous domains [5], and antibodies specific to OM are detected frequently in the sera from patients suffering from egg allergy [6]. Some research groups have reported that OM plays a more significant role in the pathogenesis of allergic reactions to egg white than other egg white proteins [7, 8], and T cell epitopes specific to OM have recently been reported [9]. The T cell receptor (TCR) of CD4$^+$ T lymphocytes recognizes immunogenic peptide sequences bound within the groove of human leucocyte antigen (HLA) class II molecules [10]. Such recognition results in a signal that is transmitted through the TCR, activates intracellular signal transduction pathways, and culminates in the proliferation and differentiation of CD4$^+$ T cells into effector cells that execute many functions [11]. Proposed models of the interaction of TCR with HLA molecules orientate the diverse complementarity determining region (CDR) 3 loops above the diverse peptides and the less diverse CDR1 and CDR2 loops above the correspondingly less diverse HLA molecules [12–16]. We hypothesize that the mechanisms of allergic reactions

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originate from the recognition of the antigen presented by specific HLA class II molecules. We established OM-specific T cell lines (TCLs) and T cell clones (TCCs) from four Japanese AD patients with egg-white allergy in order to identify T cell epitopes on OM, antigen-presenting HLA class II molecules, surface-marker phenotypes, and the usage of the CDR3 loops of the TCR α and β chains of the TCCs.

Materials and methods

Patients

The diagnosis of allergy to hen's egg-white was based on clinical symptoms, antigen challenge test results and CAP-RAST [17] results to hen's egg-white and ovomucoid (Table 1). Patients TM and YN had shown symptoms of immediate type hypersensitivity such as systemic urticaria and severe cough, which occurred within 30 min after antigen challenge, and their degree of total IgE and CAP-RAST results for egg white and OM were high. Patients IH and YT had shown symptoms of delayed type hypersensitivity (DTH), such as systemic eczema, which occurred more than 24 h after antigen challenge, and their degree of total IgE and CAP-RAST for egg white and OM were low.

Synthesis of OM-derived peptides

OM-derived peptides were synthesized using the multiple method (Melbourne, Australia) based on Fmoc strategy. Peptides of 14–22 residues were overlapped by 11–15 residues, and corresponding to the entire sequences of OM. All peptides were purified by C18 reverse-phase HPLC (Gilson, Middleton, WI, USA). The primary structures of peptides used in this study are shown in Fig. 1.

Generation of antigen-specific TCLs and TCCs

OM-specific TCLs were established from peripheral blood mononuclear cells (PBMCs) from four patients with egg-white allergy. TCLs were generated by stimulating PBMC (1.5×10^5/well) with a mixture of 23 synthetic OM-derived peptides at a final concentration of 0.1 μM each, in the RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 96-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). After 7 to 9 days, irradiated (3000 cGy) autologous PBMC (1.5×10^5/well) pulsed with OM peptide mixture (1 μM each for 5 h), human recombinant interleukin (rIL)-2 (50 U/mL) (Genzyme, Cambridge, MA, USA) and human rIL-4 (10 U/mL) (Biosourse International,

Table 1. Clinical features and HLA class II haplotypes of the subjects studied

Pt	Age	Sex	Symptom	Total IgE (IU‡/mL)	CAP-RAST for egg white (UA§/mL)	CAP-RAST for OM (UA§/mL)	HLA-DRB1*	HLA-DRB3*	HLA-DRB4*	HLA-DRB5*	HLA-DQA1*	HLA-DQB1*
TM	8 m	M	AD†	2239.5	< 100	< 100	0802/ 0901	–/–	–/0101	–/–	0301/ 0301	0302/ 0303
YN	10 m	M	AD†	2430.7	< 100	< 100	0406/ 0406	–/–	0101/ 0101	–/–	0301/ 0301	0302/ 0302
IH	1.1 yr	M	AD†	30.7	< 0.34	< 0.34	0901/ 1403	–/0101	0101/–	–/–	0301/ 0501	0301/ 0303
YT	6 m	M	AD†	8.4	1.2	0.6	0405/ 1502	–/–	0101/–	–/0102	0301/ 0103	0401/ 0601

†Atopic dermatitis. ‡International unit. §Unit of allergen-specific IgE.

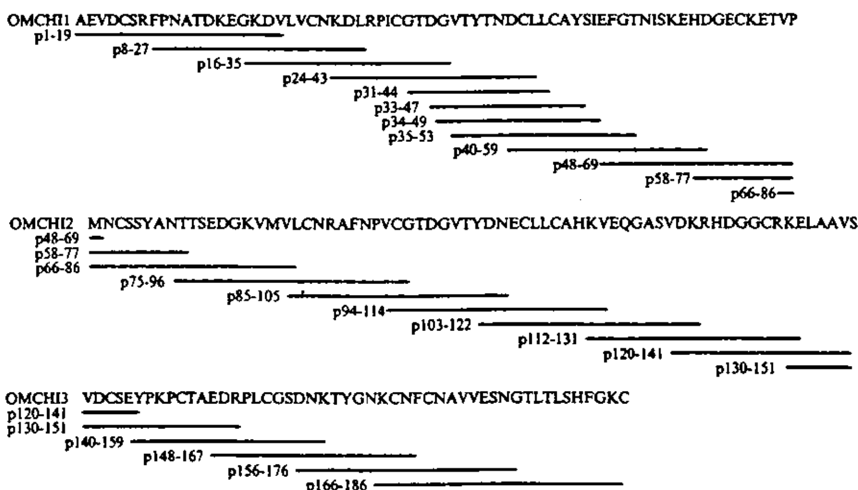

Fig. 1. Schema of the synthetic peptides. OM consists of three tandem homologous domains (OMCHI1-OMCHI3). Lines represent synthetic peptides composed of 14–22 residues overlapping each other by 11–15 residues. Numbers refer to the location of the residues in the primary structure of OM.

Camario, CA, USA) were added only to culture wells that were carrying T cell blasts and were thus maintained for another 7 days. Aliquots of growing cultures were combined as a bulk TCL for each patient, and reactivity against the overlapping peptides was determined. Cloning was done in Terasaki plates (Nunc, Roskilde, Denmark), by limiting dilution at 0.3 cells per well in the presence of irradiated (3000 cGy) autologous PBMC (3×10^4/well) pulsed with OM peptide mixture (1 μM), human rIL-2 (50 U/mL) and human rIL-4 (10 U/mL), in the same medium described above. Growing microcultures (approximately 5% of all culture wells) were then expanded at weekly intervals, first in a 96-well plate and then in a 24-well plate by feeding irradiated feeder cells (1.5×10^6/well) pulsed with OM peptide mixture (1 μM), in the presence of rIL-2 and rIL-4.

Antigen-induced proliferative responses of the TCLs and TCCs

Antigen-induced proliferation of the TCLs and TCCs was assayed by culturing the T cells (3×10^4/well) in 96-well flat-bottomed culture plates in the presence of the soluble OM peptide mixture (1 μM each), respective overlapping peptides (1 μM), or OM protein (2.5 or 25 mg/mL) and 3000 cGy-irradiated autologous PBMC (1.5×10^5/well). Cells were cultured for 72 h, in the presence of 1 mCi/well of [^{3}H]TdR during the final 16-h period, and the incorporated radioactivity was measured by liquid scintillation counting. To determine restriction molecules for antigen presentation, the TCLs and TCCs were cultured with irradiated autologous PBMC, with or without anti-HLA class II monoclonal antibodies (mAbs) HU-4 (anti-HLA-DRB1 + DRB5 IgG2a, monomorphic) [18, 19], L243 (anti-HLA-DRB1 + DRB4 IgG2a, monomorphic, reactivity to DRB3 and DRB5 yet to be determined) [20], HU-11 (anti-HLA-DQ4 + DQ5 + DQ6 IgG2a) [21], HU-18 (anti-HLA-DQ7 + DQ8 + DQ9 IgG2a) [22], HU-46 (anti-HLA-DQ4 IgG2a) [21] and B7/21 (anti-HLA-DP IgG1, monomorphic) [23]. The percentage inhibition was calculated using the following formula: {1 − (Value obtained with a peptide and a mAb − that with medium alone)/(Value obtained with a peptide and without a mAb − that with medium alone)} × 100. Other experiments were done in which PBMC of two donors with one haplotype-shared and one donor with irrelevant HLA haplotypes were used as antigen presenting cell (APC). In this experiment, allogeneic PBMC (3×10^5/well) were cultured in plates with peptides (1 μM each) in the culture medium, for 2 h at 37 °C, then excess peptides and non-adherent cells were removed by gently washing the plates three times with RPMI 1640 media containing 3% human serum. The remaining adherent cells were irradiated at 3000 cGy and used as APC.

HLA typing

HLA class II (DR, DQ) alleles were determined by hybridization of HLA-DR, DQ genes amplified by polymerase chain reaction with sequence-specific oligonucleotide probes distributed in the 11th International Histocompatibility Workshop, as described elsewhere [24]. The nomenclature of the HLA-DR, DQ alleles was according to the WHO Nomenclature Committee for factors of the HLA system [25].

Flow cytometric analysis

Fluorescein-isothiocyanate (FITC)-conjugated mAbs anti-Leu2a/CD8, anti-TCR-α/β-1-WT31 (Becton Dickinson, San Jose, CA, USA), anti-TCR-γ/δ I (Endogen, Woburn, MA, USA), phycoerythrin (PE)-labelled anti-Leu4/CD3 and anti-Leu3a/CD4 (Becton Dickinson) were used to analyse the phenotype of our TCCs by double colour staining. TCCs were washed with PBS, and 10^5 cells were incubated and double-stained with CD4 (PE conjugated)/CD8 (FITC conjugated) or CD3 (PE conjugated)/anti-TCR-α/β-1 WT31 (FITC conjugated) or CD3 (PE conjugated)/anti-TCR-γ/δ (FITC conjugated). Stained cells were analysed using a FACScan instrument (Becton Dickinson). Forward scatter threshold was set to exclude only debris in the preparation.

First-strand-cDNA synthesis and PCR amplification

The total RNA was extracted from TCCs using the single-step guanidinium-phenol-chloroform extraction method [26]. Single-stranded-cDNA synthesis was carried out using 1.2 to 1.8 μg of total RNA with oligo (dT) and avian myeloblastosis virus reverse transcriptase. TCR α and β cDNAs were directly subjected to DNA amplification by polymerase chain reaction (PCR) using Amplitaq DNA polymerase (Perkin Elmer, Norwalk, CT, USA) with oligonucleotide primers complementary to TCR V and C region sequences [27] at a final concentration of 0.25 μM. Reaction mixtures were incubated in a DNA thermal cycler (model No. PJ1000, Perkin Elmer) under the following conditions: denaturation at 93 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, with a further 5-s extension after each cycle.

Cloning and sequence analysis of TCR α and β chains

The PCR reaction products were cloned into the TA cloning vector pCR II™ (Invitrogen Corporation, San Diego, CA, USA) and sequenced by the enzymatic chain termination method [28], using a sequence version 2.0 kit (United States Biochemical Corporation, Cleveland, OH, USA). For each of the TCR α and β chains studied, three independent pCR II™ cloning vectors were sequenced to identify possible sequence errors caused by nucleotide misincorporation by the Taq polymerase. Sequences were compared with gene data bank entries (GenBank, Los Alamos, NM, USA; EMBL, Heidelberg, Germany) and assigned to available published TCR V and J segments [29–32]. Virtually all TCR V transcripts have the amino acid sequence Y-(L/F)-C-A (α chain) or Y-(L/F)-C-A with two additional serine residues in most cases (β chain) at the 3' end of the V gene segment and the motif F-G-X-G-T within the J segment [33]. The CDR3 length was taken as the number of amino acids between the last germline-encoded residue of the V segment and the conserved phenylalanine residue.

Results

Polyclonal TCLs specific to OM

TCLs specific to OM were established from PBMCs of four patients with hen's egg white allergy. The cell lines were tested for their proliferative response to a panel of overlapping synthetic peptides corresponding to the amino acid sequence of the entire OM. TCL established from patient TM proliferated in

the presence of OM peptides 8–27, 35–53, 40–59, 85–105, 103–122, 156–176 and 166–186 (Fig. 2a). TCL from YN proliferated in the presence of OM peptides 40–59, 48–69, 75–96, 120–141 and 130–151. TCL from IH proliferated in the presence of OM peptides 75–96, 85–105, 120–141, 130–151 and 166–186. TCL from YT proliferated in the presence of OM peptides 120–141 and 130–151.

Antigen-presenting HLA molecules of TCLs specific to OM

TCLs from patients TM and YT were inhibited by HU4 (anti-DRB1 + 5 mAb, 41.7% and 7.6%, respectively) and L243 (anti-DRB1 + 4 mAb, 61.0% and 99.5%, respectively). TCL from YN was inhibited by HU11 (anti-DQ4 + DQ5 + DQ6 mAb, 17.0%) and HU18 (anti-DQ7 + DQ8 + DQ9 mAb, 14.3%), and TCL from IH was inhibited by L243 (anti-DRB1 + 4 mAb, 87.9%) (data not shown).

TCCs specific to OM

TCCs specific to OM were established from patients TM, YN, IH and YT; each was cloned from the TCLs mentioned above (Fig. 2b). TCC 'TM1.3' recognized OM peptides 35–53 and 40–59, whereas TCC 'TM1.4' recognized OM peptide 166–186. TCC 'YN1.5' recognized OM peptides 40–59 and 48–69, and TCC 'IH3.3' and 'YT6.1' recognized OM peptides 120–141 and 130–151.

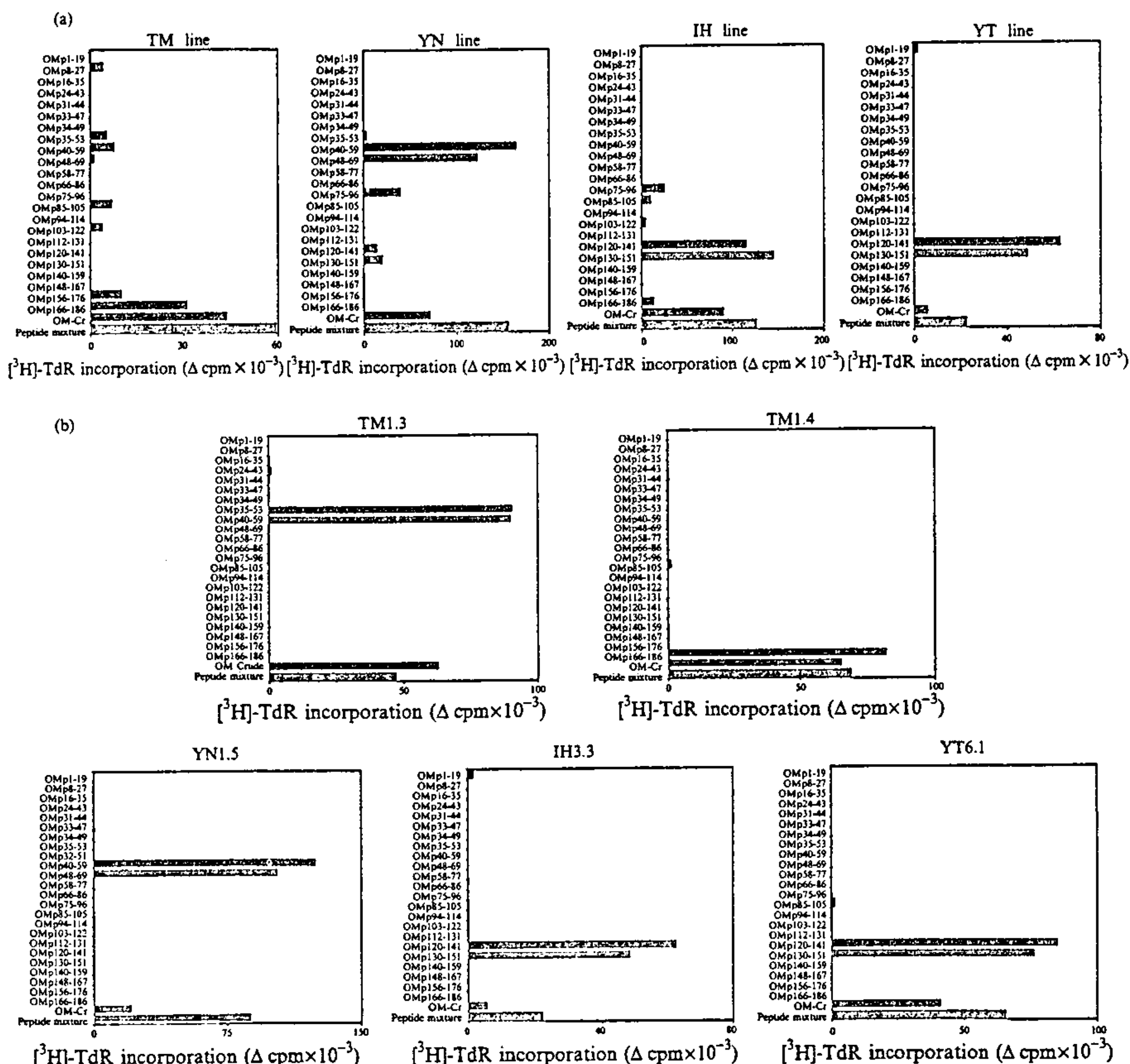

Fig. 2. Proliferative responses of four short-term T cell lines (a) and T cell clones (b) to 11- to 15-residue-overlapped synthetic peptides derived from OM. Peptides are designated by the amino acid residue numbers of the amino- and carboxyl-termini. Values shown are the mean cpm of duplicate cultures after subtraction of the mean cpm obtained from cultures without peptides. The standard error (SE) of duplicate cultures was < 20%.

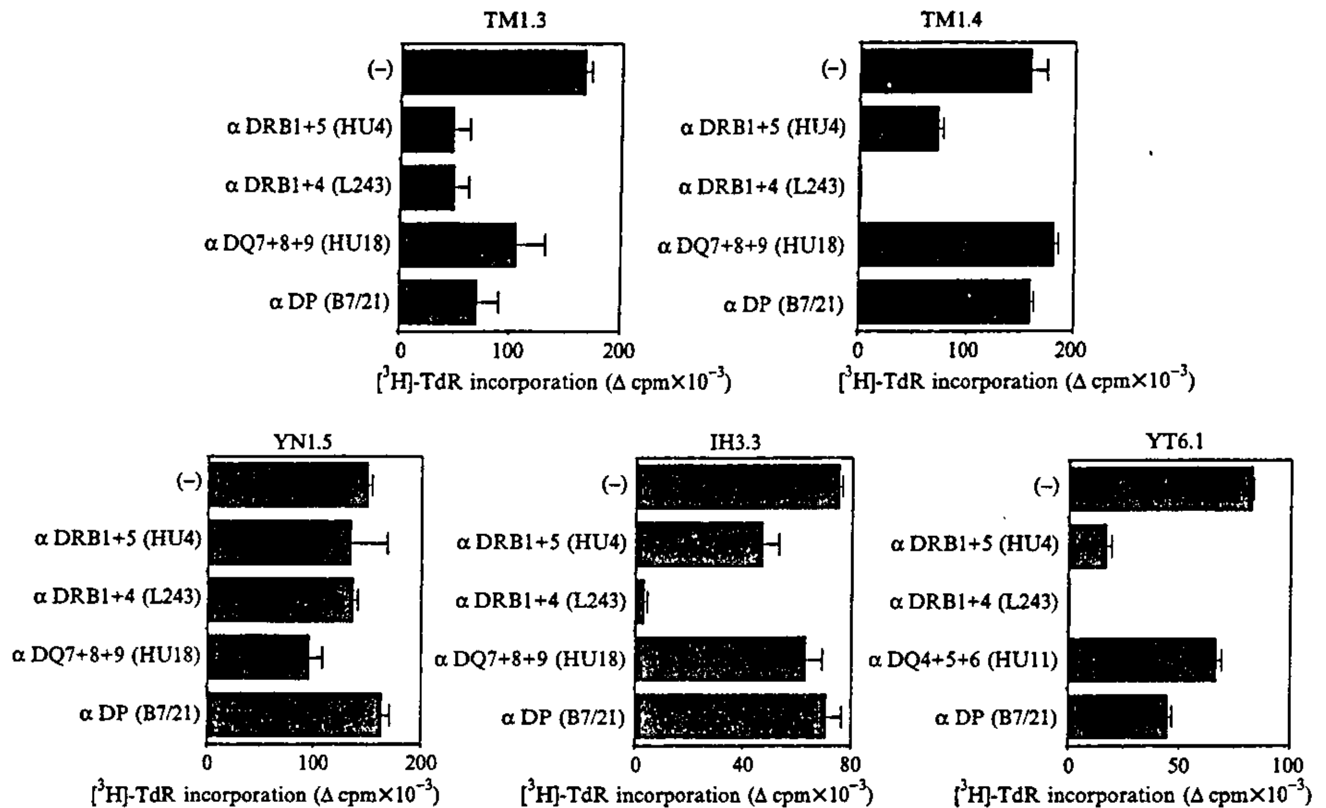

Fig. 3. Proliferation inhibition of five short-term T cell clones using anti-HLA class II mAbs. T cells (3×10^4/well) were incubated with HU4 (anti-HLA-DRB1 + DRB5 mAb), L243 (anti-HLA-DRB1 + DRB4 mAb), HU11 (anti-HLA-DQ4 + DQ5 + DQ6 mAb), HU18 (anti-HLA-DQ7 + DQ8 + DQ9 mAb), HU46 (anti-HLA-DQ4 mAb) and B7/21 (anti-HLA-DP mAb), and irradiated autologous PBMCs (1.5×10^5/well), with or without OM peptides (1 μM each). Results are expressed as the geometric mean of triplicate determinations ± standard error. All mAbs were tested at a final dilution of 1:200.

Antigen-presenting HLA molecules of TCCs specific to OM

TCCs 'TM1.3', 'TM1.4' and 'YT6.1' were inhibited by HU4 (anti-DRB1 + 5 mAb, 71.3%, 54.0% and 7.6%, respectively) and L243 (anti-DRB1 + 4 mAb, 70.5%, 99.4% and 99.5%, respectively). TCC 'YN1.5' was inhibited by HU18 (anti-DQ7 + DQ8 + DQ9 mAb, 35.7%), and 'IH3.3' was inhibited by HU4 (anti-DRB1 + 5 mAb, 37.3%) and L243 (anti-DRB1 + 4 mAb, 95.6%) (Fig. 3).

HLA recognition patterns of TCCs specific to OM

TCCs 'TM1.3' and 'TM1.4' proliferated in response to OM peptides presented by allogeneic PBMCs sharing HLA-DRB1*0901. TCC 'YN1.5' proliferated in response to OM peptides presented by allogeneic PBMCs sharing HLA-DQB1*0302, TCC 'IH3.3', HLA-DRB4*0101, and TCC 'YT6.1', HLA-DRB1*0405 (Fig. 4).

TCR α and β gene usage and comparison of CDR3 loop of TCCs specific to OM

Sequence analysis of the TCR α and β gene usage of the TCCs specific to OM revealed marked heterogeneity (Fig. 5). TCCs 'TM1.3' and 'TM1.4' were cloned from the same TCL from patient TM; nevertheless, their TCR α and β gene usages were different (TCRAV19S1 and AJ48, TCRAV8S1 and AJ41; TCRBV22S1 and BJ1S6, TCRBV3S1 and BJ2S2, respectively). Interestingly, TCCs 'YN1.5', 'IH3.3' and 'YT6.1' have the same TCR V β gene usage (TCRBV2S1). TCCs 'IH3.3' and 'YT6.1' also have the same TCR J β gene usage (TCRBJ1S1). Moreover, the CDR3 loop of the TCCs specific to OM was composed of heterogenous amino acid residues.

Surface-marker phenotypes

All the TCLs and TCCs were of $CD3^+$, $CD4^+$, $CD8^-$, $\alpha\beta TCR^+$, $\gamma\delta TCR^-$ phenotype (data not shown).

Discussion

Our previous study showed that seven major T cell epitopes were identified by TCCs specific to β-lactoglobulin [34]; in this study, several epitopes were identified by five OM-specific TCCs. Four T cell epitopes specific to OM (OM41-56, OM71-84, OM131-144 and OM171-186) have recently been reported [9]. Our six OM peptides (35–53, 40–59, 48–69, 120–141, 130–151, 166–186) have three (OM41-56, OM131-144 and OM171-186) of their four epitopes overlapping, indicating that T cell epitopes specific to OM are identical regardless of species. Even though Zhang et al. [35] reported that there were significantly more activities of IgG and IgE binding to the third domain of OM than to the first and second domains using egg-allergy patients' sera, Cooke et al. [36] mentioned that IgE- and IgG-binding epitopes specific to OM existed in all OM domains. Our investigation also showed that T cell epitopes specific to OM existed in all OM domains. Our previous report showed that the TCCs specific to β-lactoglobulin tend to associate with

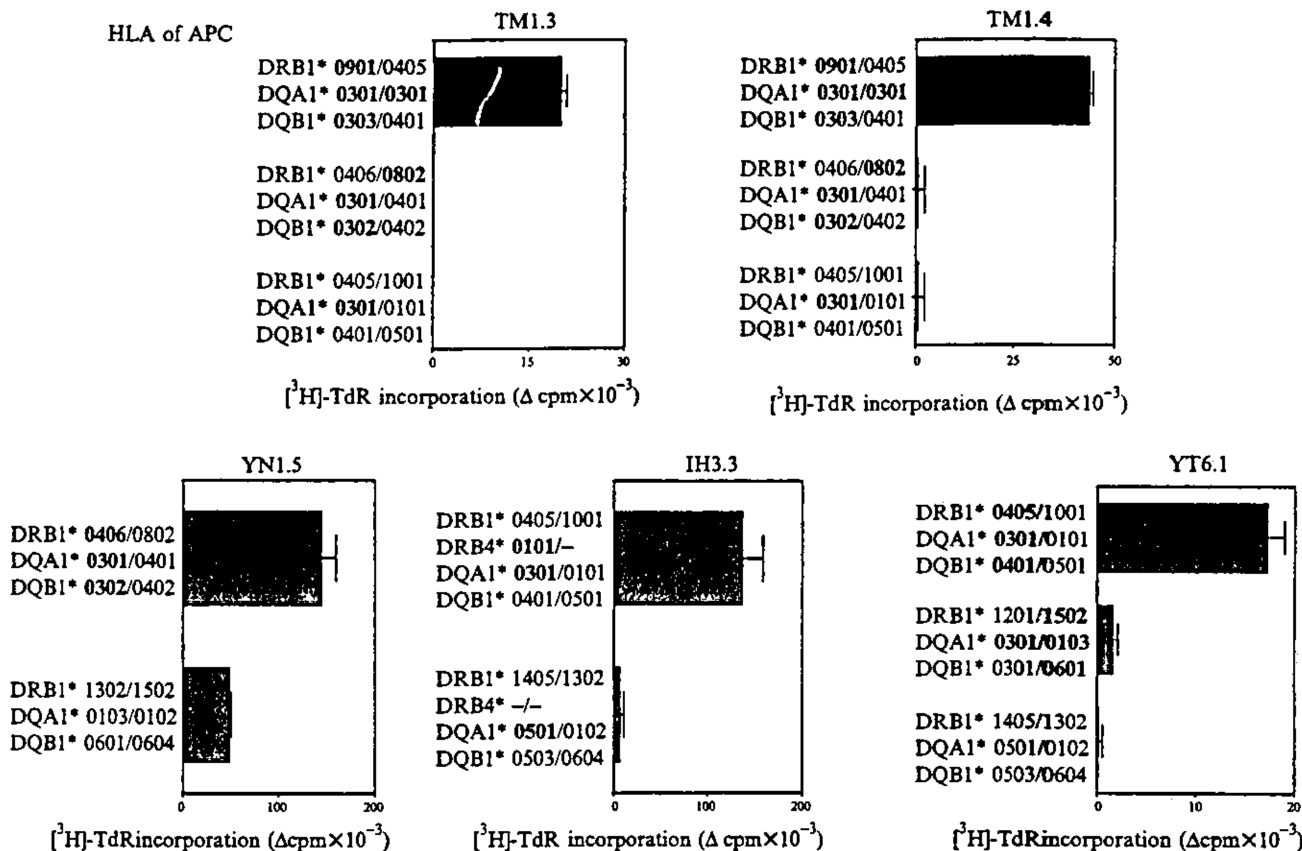

Fig. 4. Antigen presentation to our T cell clones by allogeneic PBMCs. Allogeneic PBMCs (3×10^5/well) were cultured in a 96-well plate with 1 μM of the OM peptide mixture for 2 h at 37 °C; excess peptides and non-adherent cells were removed by gently washing the plate three times with the RPMI 1640 medium containing 3% human serum. The remaining adherent cells were irradiated at 3000 cGy and used as APC. T cells (3×10^4/well) were then added to the culture plate. Shared HLA haplotypes are indicated in bold letters. Results are expressed as the geometric mean of triplicate determinations ± standard error.

the HLA-peptide complex presented by HLA-DRB1*0405 [37]. The antigen-presenting molecules used by TCCs specific to OM were different for each TCC.

Although the diversity-generating mechanisms for antibodies and TCR are very similar, there is much more diversity in TCR J segments and N additions at CDR3 [12], and apparently less variation at CDR1 and CDR2 [38]. Sequence analysis of the TCR α and β gene usage of our TCCs specific to OM showed marked heterogeneity. 'TM1.3' and 'TM1.4' were cloned from the same TCL from patient TM; nevertheless, they recognized different peptides, and their usage of the CDR3 loops of the TCR α and β chains was different. These peptides were presented by the same HLA molecule (DRB1*0901). Only three to five amino acids within a peptide bound to the groove of HLA molecules face outwards into the solvent for potentially direct contacts with TCR amino acids [39–41]. Similarly, although many amino acids constitute the framework of the TCR α and β chains, only a few amino acids have been confirmed to directly contact amino acids [42–47].

As shown in Fig. 5, each of the CDR3α and CDR3β loops of TCCs has the positively charged residues 'Q', 'K', 'N' and 'R' and the negatively charged residues 'E' and 'D'. Considering that peptide motifs bind to HLA class II molecules as reported previously [48–50], the electrically charged residue (positive or negative) on the CDR3α and the CDR3β loops of TCR of TCC may form ionic bonds with a charged residue on the HLA class II molecule-peptide complex. Our previous report also showed that the negatively charged residue 'E' existed in the HLA-DRB1*0405-β-lactoglobulin peptide complex, and the positively charged residue 'K' existed in the CDR3α loop [37].

The antigen-presenting molecules of TCCs 'IH3.3' and 'YT6.1' from patients IH and YT were different, but the TCCs recognized the same OM peptides 120–141 and 130–151. Although the CDR3 loop of TCCs 'YN1.5', 'IH3.3' and 'YT6.1' was composed of heterogenous amino acid residues, the TCCs have the same TCR Vβ gene usage, and 'IH3.3' and 'YT6.1' also have the same TCR Jβ gene usage. Moreover, it is an attractive finding that patients IH and YT had shown symptoms of DTH. These data suggest that antigen recognition by a specific TCR is closely associated with the characteristics of each patient's symptoms.

Further experiments investigating other food antigens will clarify the correlation among antigens, antigen-presenting molecules and TCR, and assess immunopathologies of patients with AD. Furthermore, these studies will lead to the development of therapeutic approaches for AD.

(a)

T cell clone	TCR gene segment		TCRAV	CDR3	TCRAJ
TM1.3	AV19S1	AJ48	CCCAGAGACTCTGCCGTCTACATCTGTGCTGTC P R D S A V Y I C A V	CTCTTTCAGGGAGCCCAGAAGCTGGTA L F Q G A Q K L V	TTTGGCCAAGGAACCAGGCTGACTATCAAC F G Q G T R L T I N
TM1.4	AV8S1	AJ41	CCTGAAGACTCGGCTGTCTACTTCTGTGCAGCA P E D S A V Y F C A A	AGTGGGGCTTCCGGGTATGCACTCAAC S G A S G Y A L N	TTCGGCAAAGGCACCTCGCTGTTGGTCACA F G K G T S L L V T
YN1.5	AV4	AJ42	CTGAGAGACACTGCTGTGTACTATTGCATCGTC L R D T A V Y Y C I V	AGGGGAGGAAGCCAAGGAAATCTCATC R G G S Q G N L I	TTTGGAAAAGGCACTAAACTCTCTGTTAAA F G K G T K L S V K
IH3.3	AV2S1	AJ14	CTCAGTGATTCAGCCACCTACCTCTGTGTGGTG L S D S A T Y L C V V	AACGTAGGGAGTGCTTCCAAGATAATC N V G S A S K I I	TTTGGATCAGGGACCAGACTCAGCATCCGG F G S G T R L S I R
YT6.1	AV7	AJ37	ACTGGGGACTCAGCCACCTACCTCTGTGCC T G D S A T Y L C A	CCCGCCGGAGTGAGCAACACAGGCAAACTAATC P G G V S N T G K L I	TTTGGGCAAGGGACAACTTTACAAGTAAAA F G Q G T T L Q V K

(b)

T cell clone	TCR gene segment		TCRBV	CDR3	TCRBJ
TM1.3	BV22S1	BJ1S6	CTGGAGGACTCAGCCATGTACTTCTGTGCCAGC L E D S A M Y F C A S	ACCCGCATTCGTGGGGGTTCCGATGGCTCACAC T R I R G G S D G S H	TTCGGTTCGGGGACCAGGTTAACCGTTGTA F G S G T R L T V V
TM1.4	BV3S1	BJ2S2	ACCAACCAGACATCTATGTACCTCTGTGCC T N Q T S M Y L C A	ACCTATAGCGGGGAGCTGTTT T Y S G E L F	TTTGGAGAAGGCTCTAGGCTGACCGTACTG F G E G S R L T V L
YN1.5	BV2S1	BJ1S5	CCTGAAGACAGCAGCTTCTACATCTGCAGTGCG P E D S S F Y I C S A	TGGGACTCCGATCAGCCCCAGCAT W D S D Q P Q H	TTTGGTGATGGGACTCGACTCTCCATCCTA F G D G T R L S I L
IH3.3	BV2S1	BJ1S1	CCTGAAGACAGCAGCTTCTACATCTGCAGTGCT P E D S S F Y I C S A	AGAGCGGGGGGGATGAACACTGAAGCTTTC R A G G M N T E A F	TTTGGACAAGGCACCAGACTCACAGTTGTA F G Q G T R L T V V
YT6.1	BV2S1	BJ1S1	CCTGAAGACAGCAGCTTCTACATCTGCAGTGCT P E D S S F Y I C S A	AGTATGCGACAGGGGGCAGTGGTAGCTTTC S M R Q G A V V A F	TTTGGACAAGGCACCAGACTCACAGTTGTA F G Q G T R L T V V

Fig. 5. TCR α (a) and β (b) gene usages and CDR3 loops of the TCCs. Nucleotide and deduced amino acid sequences of junctional regions of the TCR of T cell clones specific to OM are shown. The underlined CDR3 sequences are charged residues.

Acknowledgements

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Cutaneous Biology

Oral administration of persimmon leaf extract ameliorates skin symptoms and transepidermal water loss in atopic dermatitis model mice, NC/Nga

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Summary

Background We have previously shown that persimmon leaf extract and its major flavonoid constituent, astragalin, inhibited histamine release by basophils and that oral administration of these substances prior to the onset into an atopic dermatitis (AD) model mouse, NC/Nga, prevented development of dermatitis.

Objectives This study was designed to assess the clinical therapeutic effect of persimmon leaf extract and astragalin in NC/Nga mice suffering from dermatitis and the dose–response preventive effects of persimmon leaf extract on dermatitis and transepidermal water loss (TEWL).

Methods The efficacy of persimmon leaf extract or astragalin in NC/Nga mice was judged by measurement of skin severity, scratching behaviour, serum IgE levels or TEWL.

Results Oral administration of persimmon leaf extract (250 mg kg^{-1}) or astragalin (1·5 mg kg^{-1}) for 4 weeks into NC/Nga mice with overt dermatitis resulted in a decrease in the severity of the condition. The preventive effect of persimmon leaf extract on the dermatitis was dose-dependent and continuous intake of persimmon leaf extract significantly decreased its onset and development. In addition, TEWL was also suppressed at a persimmon leaf extract dose of 250 mg kg^{-1}. No significant adverse reaction by these substances could be observed.

Conclusions These observations suggest that persimmon leaf extract or the flavonoid astragalin may be alternative substances for the management of AD.

Key words: astragalin, atopic dermatitis, mice, persimmon leaf extract, transepidermal water loss

The persimmon *Diospyros kaki* Thunberg (Ebenanceae) grows in China, Korea and Japan. It was found that tea brewed from the leaves of this tree had a beneficial effect on haemostasis, diuresis, constipation and hypertension. Kaempferol-3-glucoside (astragalin) from persimmon leaves was found to possess hypotensive action through its suppression of angiotensin-converting enzyme activity.[1,2] In a previous study, we showed that persimmon leaf extract inhibited histamine release from human basophilic cell line KU812.[3] In addition, we also reported inhibitory effects of persimmon leaf extract and astragalin (considered to be the primary ingredient for the action of persimmon leaf extract) on the development of dermatitis and IgE elevation by NC/Nga, atopic dermatitis (AD) model mice.[4] In this report the pharmacological effects of these materials, particularly their efficacy on the severity of dermatitis in mice and the potential to prevent its onset and development and also on transepidermal water loss (TEWL) were studied.

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Materials and methods

Chemicals

Persimmon leaf from Shi-chuan, China was purchased at a retail shop in Japan. Astragalin (Kaempferol-3-glc)

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was purchased from Extrasynthese (Genay, France). The MF® diet was obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan).

Preparation of persimmon leaf extract

The persimmon leaf was steeped in boiling distilled water for 5 min and filtered through a nylon mesh (pore size 40 μm). After centrifugation at 5000 *g* for 20 min at 4 °C, the supernatant of the persimmon leaf extract was freeze-dried. The contaminated endotoxin level in persimmon leaf extract and astragalin was less than 1 ng mL^{-1}. Astragalin content was determined by means of high-performance liquid chromatography (HPLC) at 598 mg of astragalin per 100 g of persimmon leaf extract.

Continuous feeding of persimmon leaf extract or astragalin in NC/Nga mice and evaluation of severity in skin lesions

Four-week-old NC/Nga mice (conventional grade) were obtained from Japan SLC Inc. (Shizuoka, Japan) and housed at 23 ± 3 °C in 55 ± 15% humidity with 12 h/12 h of light/dark cycle (light on 07.00–19.00).

I: Therapeutic trial. In order to see the therapeutic effects of persimmon leaf extract or astragalin on the dermatitis, NC/Nga mice were kept under conventional circumstances until 13 weeks of age. The severity of skin symptoms was then evaluated as described later and divided into three groups (five mice in each group). Five mice were provided with one of the following diets *ad libitum*: control diet (MF diet), persimmon leaf extract diet (MF diet containing 0·125% persimmon leaf extract, 250 mg kg^{-1}) or astragalin diet (MF diet plus 0·00075% astragalin, 1·5 mg kg^{-1}) for the following 4 weeks.

II: Prevention study. In the second experiment mice with no skin symptoms at the age of 5 weeks were divided into four groups (five mice in each group) and then were provided with one of the following diets *ad libitum*: control diet (MF diet) or persimmon leaf extract diet (6, 50 or 250 mg kg^{-1}) until 16 weeks.

The severity of dermatitis was assessed once a week with the following scoring system by observers who were blinded to the treated group. Symptoms were evaluated by skin dryness, eruption and wound on the three parts of the body: ear, face and head, and back. Each symptom was graded from 0 to 3 (no symptom, 0; mild, 1; moderate, 2; and severe, 3).

Measurement of immunoglobulin concentrations

Blood was taken from retro-orbital vessels after mice were anaesthetized and serum was obtained by centrifugation at 1000 *g* for 5 min at 4 °C and stored at −80 °C until use. The serum IgE level was measured by means of an enzyme immunoassay using monoclonal antibodies (Yamasa Shoyu Co. Ltd, Chiba, Japan). The serum level of other immunoglobulins, including IgM, IgG1, IgG2a, IgG2b and IgG3 were also measured using mAb-Based Mouse immunoglobulin Isotyping kit (PharMingen, San Diego, CA, U.S.A.).

Evaluation of scratching behaviour

The frequency of scratching behaviour of 13- and 17-week-old NC/Nga mice, such as scratching of the nose, ears and dorsal skin with the hind paws, was measured during a 20-min period by observers who were blinded to the treated group, according to the previous report.[5] Licking of the belly and dorsal skin during grooming was disregarded. Each occurrence of scratching of the head, neck, dorsal skin, ears and nose was scored to obtain the maximum score.

Measurement of transepidermal water loss

TEWL was assessed on the dorsal skin of five NC/Nga mice at the age of 16 weeks using Tewameter TM210 (Courage+Khazaka Electronic GmbH, Cologne, Germany) according to previous reports.[6,7] The temperature ranged from 21 °C to 22 °C and the relative humidity was maintained at 50–55%. Measurements were recorded when TEWL readings were stabilized at approximately 45 s after the probe was placed on the skin.

Evaluation of adverse reactions of persimmon leaf extract and astragalin

Serum was obtained from five NC/Nga mice in each group on the last day of the test period and biochemical parameters, including total protein, albumin, total bilirubin, aminotransaminase (aspartate aminotransferase and alanine aminotransferase), choline esterase, lactate dehydrogenase, creatine phosphokinase, blood urea nitrogen, creatinine, uric acid, amylase, blood sugar, total cholesterol and triglyceride, were measured. Body weight was also assessed at 13 weeks

and at 17 weeks of age when NC/Nga mice were administered with the control diet, persimmon leaf extract diet (250 mg kg^{-1}) or astragalin diet (1·5 mg kg^{-1}).

Statistics

The statistical significance of differences in the skin severity score between treatment groups and control group was assessed using the Kruskall–Wallis non-parametric test and *post hoc* testing was performed using the Mann–Whitney test. Differences in the other parameters between treatment groups and control group were analysed using one-way analysis of variance followed by Scheffer's *F* analysis.

Results

Oral administration of persimmon leaf extract or astragalin suppresses the severity of established dermatitis and scratching behaviour in NC/Nga mice

The NC/Nga mouse spontaneously develops eczema with age under conventional circumstances.[8,9] Representative skin and its histological feature is shown in Figure 1. Indeed, development of dermatitis was observed in NC/Nga mouse under conventional circumstances but not under specific pathogen-free circumstances. Histopathological examination showed that thickening of the epidermis, hyperkeratosis and the infiltration of inflammatory cells were found in NC/Nga mouse under conventional circumstances. Five mice with dermatitis were given orally persimmon leaf extract (250 mg kg^{-1}), astragalin (1·5 mg kg^{-1}) or a control diet starting at 13 weeks of age until 17 weeks. The severity of dermatitis was evaluated weekly. The artificial score of skin severity from each group was similar (5·6 ± 1·7, control; 5·4 ± 1·6, persimmon leaf extract; 5·8 ± 1·5, astragalin) before the initiation of the treatment (Fig. 2). The intake of persimmon leaf extract (P = 0·039) or astragalin (P = 0·052) remarkably inhibited the appearance of skin symptoms and even 2 weeks after the start of persimmon leaf extract administration the ameliorative effects were significant (Fig. 2). The mean score of skin severity from the persimmon leaf extract and astragalin groups gradually decreased and reached 2·2 ± 1·0 and 2·6 ± 0·8, respectively, within 4 weeks, compared with the control group which showed a gradual increase and remained high (7·0 ± 1·6).

The number of scratchings was also counted. At 13 weeks of age, there was no difference in the average number of scratchings among the groups; however, at 17 weeks of age the group treated with persimmon leaf extract showed a significant suppression in scratching

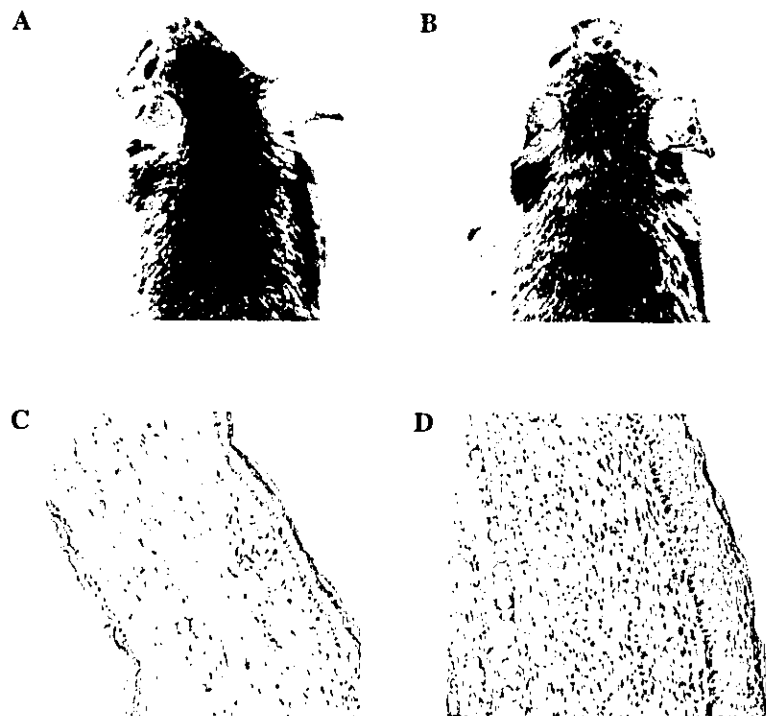

Figure 1. Clinical and histological features of NC/Nga skin: (A,C) aged 10 weeks under specific pathogen-free circumstances; (B,D) aged 10 weeks under conventional circumstance. Histology shows haematoxylin and eosin-stained section of the ear (original magnification ×10).

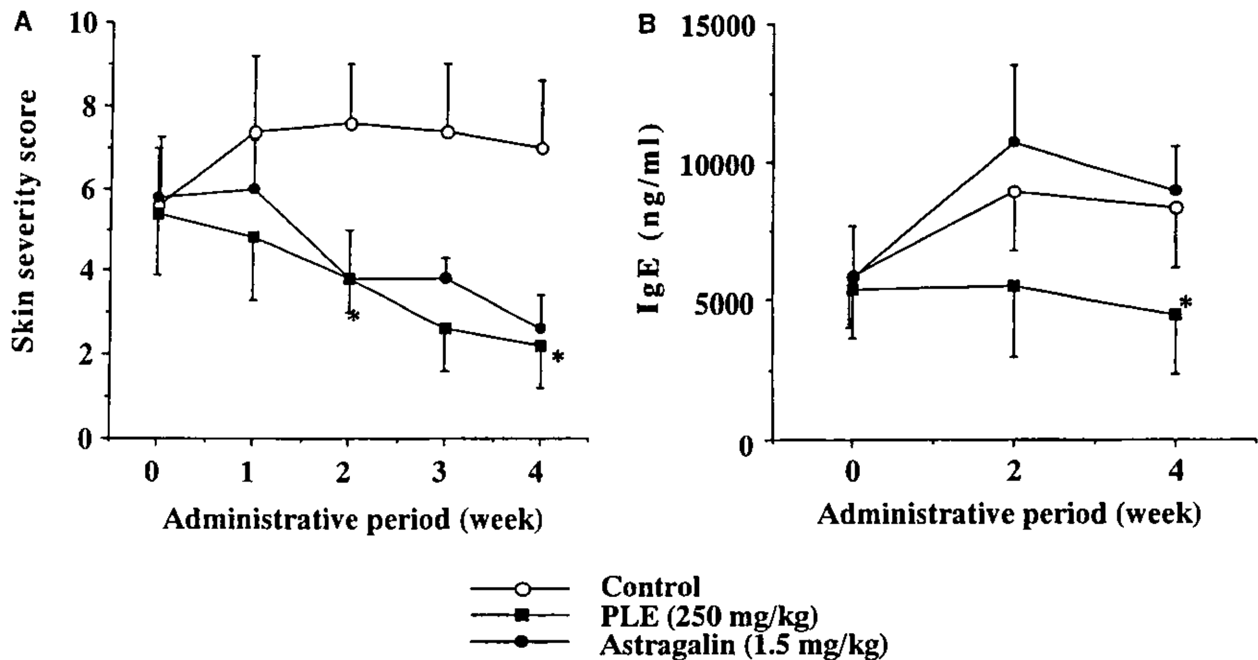

Figure 2. Effect of persimmon leaf extract or astragalin on clinical scores of skin symptoms and serum IgE levels. NC/Nga mice that had developed dermatitis at the age of 13 weeks were provided with one of the following diets *ad libitum*: Control, control diet (MF diet, $n = 5$); PLE, persimmon leaf extract diet (MF diet containing 0·125% persimmon leaf extract at 250 mg kg^{-1}, $n = 5$); or Astragalin, astragalin diet (control diet plus astragalin at 1·5 mg kg^{-1}, $n = 5$) during the following 4 weeks. (A) Clinical scores were measured weekly and (B) serum IgE levels were determined. Values represent mean ± SD. $^{*}P < 0·05$: significantly different from control diet.

behaviour (Table 1). The number of scratchings decreased fourfold after oral administration of persimmon leaf extract. Astragalin also decreased the number of scratchings, but statistically its effect was not significant. Furthermore, serum elevation of IgE level was also inhibited by persimmon leaf extract but was not inhibited by astragalin (Fig. 2). Serum concentration of IgG1 (control mice, 4886 ± 4921 µg mL^{-1}; persimmon leaf extract-treated mice, 1885 ± 1092 µg mL^{-1}; astragalin-treated mice, 1666 ± 962 µg mL^{-1}) at 17 weeks of age was decreased by oral intake of both persimmon leaf extract and astragalin. Serum concentration did not decrease in other immunoglobulin isotypes, including IgM, IgG2a, IgG2b and IgG3.

Oral administration of persimmon leaf extract dose-dependently suppresses the onset and development of dermatitis and transepidermal water loss in NC/Nga mice

In a previous report, we have demonstrated that oral intake of persimmon leaf extract at the dose of 250 mg kg^{-1} inhibited the development of dermatitis and serum IgE elevation.[4] To examine this effect of persimmon leaf extract more precisely, its dose–response effect on the clinical symptoms and TEWL as one of the parameters of impaired skin barrier was examined. The mice that had not developed dermatitis were continuously administered control diet or control diet plus 6, 50 or 250 mg kg^{-1} of persimmon leaf extract from 5 weeks of age until 16 weeks. The severity of dermatitis was then evaluated weekly. At age 8 weeks dermatitis was found in some of the mice and all control mice developed dermatitis at the age of 11 weeks (Fig. 3). The intake of persimmon leaf extract at the dose of 250 mg kg^{-1} delayed the onset and significantly decreased the severity of the dermatitis. At 11 weeks of age one of five mice exhibited signs of dermatitis, but the severity gradually decreased with age when persimmon leaf extract was administered. The dose of 6 mg kg^{-1} or 50 mg kg^{-1} of persimmon leaf extract revealed a substantial suppressive effect but the effect was not statistically significant. The representative clinical feature of NC/Nga skin at 16 weeks of age is shown in Figure 4. Finally at 16 weeks of age, TEWL was measured to evaluate the functional state of the epidermal diffusion barrier. TEWL measurements revealed that NC/Nga mice exhibited greater water loss (47·9 ± 16 g m^{-2} h^{-1}), similar to TEWL in acetone treatment.[6] The oral administration of persimmon leaf

Table 1. Scratching behaviour is suppressed by persimmon leaf extract

	No. of scratchings per 20 min	
	13 weeks	4 weeks later
Control	148 ± 113	156 ± 52
PLE (250 mg kg^{-1})	150 ± 96	*41 ± 43
Astragalin (1·5 mg kg^{-1})	140 ± 110	77 ± 64

NC/Nga mice after the onset of dermatitis at the age of 13 weeks were divided into three groups (five mice in each group). Then control diet, persimmon leaf extract (PLE) (250 mg kg^{-1}) or astragalin (1·5 mg kg^{-1}) was administered for 4 weeks. The number of scratchings per 20 min was counted prior to and 4 weeks after commencement of the diet. *Shows $P < 0·05$.

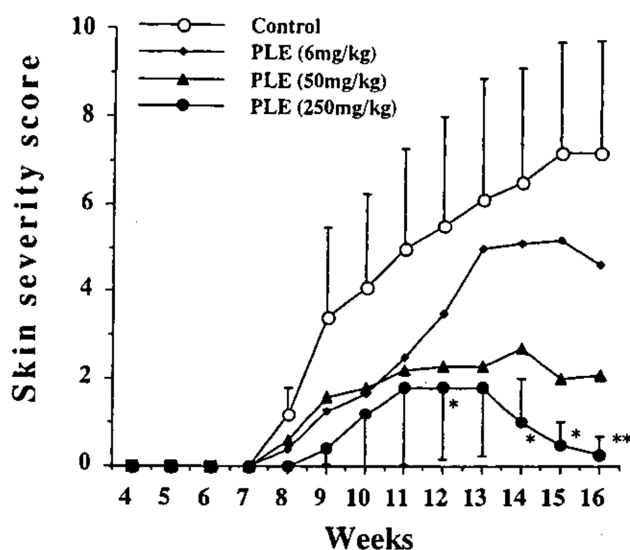

Figure 3. Dose–response effect of persimmon leaf extract (PLE) on clinical scores of skin symptoms. NC/Nga mice (five mice in each group) were orally administered control diet (Control) or control diet plus various doses (6, 50 or 250 mg kg^{-1}) of PLE from 5 weeks of age to 16 weeks. Clinical scores were measured weekly. Values represents mean ± SD. *$P < 0·05$, **$P < 0·01$: significantly different from control diet.

extract inhibited TEWL in a dose-dependent fashion as shown in Figure 5.

There is no significant adverse reaction after administration of persimmon leaf extract or astragalin

Oral administration of persimmon leaf extract or astragalin showed a significant suppressive effect on the dermatitis in NC/Nga mice. Macroscopic analysis revealed no apparent organic damage in NC/Nga mice. There was no difference in body weight among the groups in NC/Nga mice at 13 weeks of age. After 4 weeks of treatment, the change in body weights of NC/Nga mice given control diet, control diet plus persimmon leaf extract (250 mg kg^{-1}) or plus astragalin (1·5 mg kg^{-1}) was similar (from 25 ± 4 to 28 ± 5, from 24·4 ± 2·5 to 27·7 ± 2·6 and from 25·1 ± 2·8 to 28·5 ± 1·5 g, respectively). Serum markers were analysed among the treated and untreated NC/Nga groups. Those levels from control and treated mice remained within normal limits (data not shown).

Discussion

In this study we showed the clinical therapeutic efficacy of persimmon leaf extract and astragalin in NC/Nga mice and the dose–response preventive effect of persimmon leaf extract in AD-prone mice without any apparent adverse effects.

Itching is one of the major diagnostic criteria of AD and one of its most troublesome symptoms that provokes the desire to scratch.[10] Effective control of itching is believed to be one of the fundamental approaches in controlling the skin lesions. Dryness of skin is another of the representative clinical features of AD;[11] it occurs independently or is induced by allergic inflammation in the skin.[10] Maintenance of daily skin care with hydration of the skin is thus crucial for the management of AD. Indeed, scratching behaviour and TEWL were prominent in NC/Nga mice. It also has been recently shown that in NC/Nga mice TEWL was increased and the amount of ceramide of the skin was decreased at 8 weeks of age when mice were maintained under ambient laboratory conditions.[12] The administration of persimmon leaf extract led to a striking and significant effect on scratching behaviour and TEWL. From this point of view it is possible that persimmon leaf extract first leads to suppression of sensation of itching or maintains the skin barrier function, resulting in the amelioration of the dermatitis, apart from the immunological effects. To evaluate these points, further studies are required.

In the previous report, we showed that serum IgE level in 13-week-old NC/Nga mice was decreased fivefold by oral administration of persimmon leaf extract or astragalin from 5 to 13 weeks of age.[4] However, serum IgE level was not decreased by astragalin but decreased by persimmon leaf extract when mice were given each of them from 13 to 17 weeks of age. The reason for the discrepancy is unknown but it might be derived from the treatment time and period. Alternatively, the apparent difference in IgE suppression by

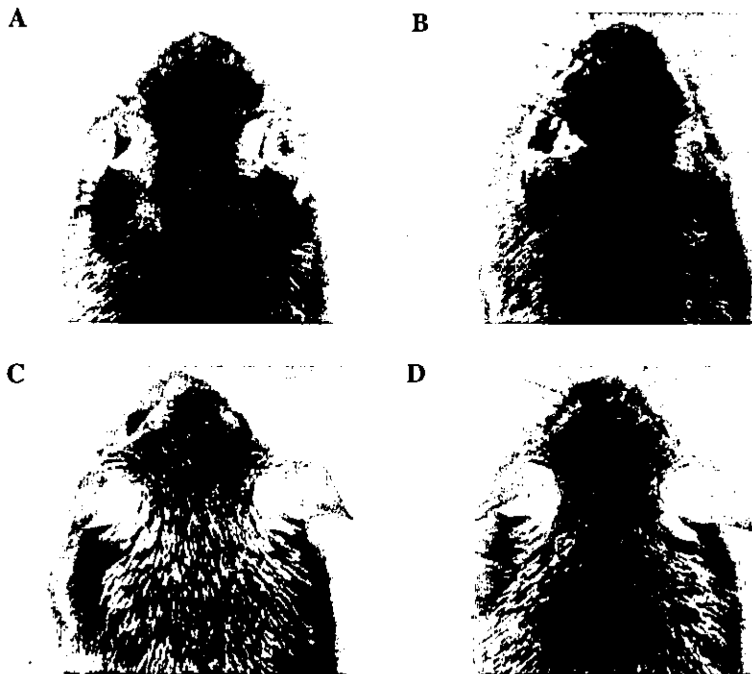

Figure 4. Representative clinical feature of NC/Nga skin. NC/Nga mice were provided with one of the following diets: (A) control diet; (B) persimmon leaf extract diet (6 mg kg^{-1}); (C) persimmon leaf extract diet (50 mg kg^{-1}); (D) persimmon leaf extract diet (250 mg kg^{-1}). The photograph was taken at 16 weeks of age.

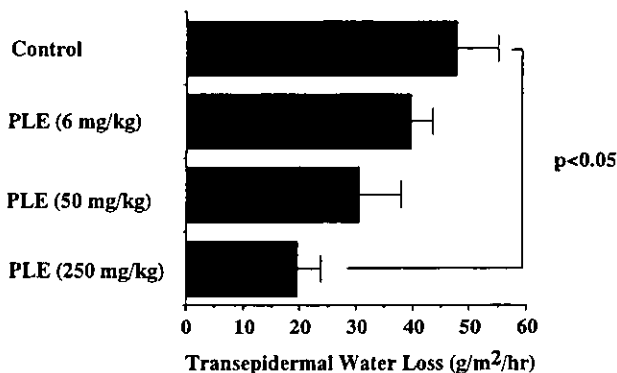

Figure 5. Persimmon leaf extract (PLE) decreases transepidermal water loss. NC/Nga mice were administered control diet (Control) or various doses of PLE as described in Figure 3. At 16 weeks of age, transepidermal water loss was measured by using Tewameter TM210. Values represents mean ± SD.

persimmon leaf extract and astragalin in this study might to be due to other constituent(s) in the extract that are primarily responsible for the IgE suppression or that it suppresses IgE production additively with astragalin. HPLC analysis of persimmon leaf extract included astragalin (0·45–0·72%), torifolin (0·32%) and isoquercitrin (0·38–0·55%), but not representative flavonoids such as quercetin, kempferol, apigenin, fisetin and luteolin.

It should be pointed out that compliance and adverse reactions of these substances were not problematic, as the weight gain was not disturbed by them. In addition, the administration of persimmon leaf extract and astragalin appeared neither to cause tissue damage through macroscopic analysis nor to induce dysfunction of organs; this was analysed by biochemical markers.

The treatment of AD is directed at symptom relief, skin hydration and the reduction of cutaneous inflammation.[13–16] The present study demonstrated that oral continuous intake of persimmon leaf extract and astragalin ameliorated severity of AD and prevented the development in AD-prone mice with no apparent side-effect. Future studies to evaluate the clinical efficacy of these materials on patients with AD or their preventive effects are essential, but we hope that these substances may constitute alternative or complementary medicine for AD and that this evidence contributes to the management of AD.

Acknowledgments

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