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Serum measurement of thymus and activation-regulated chemokine/CCL17 in children with atopic dermatitis: elevated normal levels in infancy and age-specific analysis in atopic dermatitis

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Elevated blood levels of thymus and activation-regulated chemokine (TARC)/CCL17 have been observed in atopic dermatitis (AD) and may serve as a new biomarker for AD. However, the normal levels, especially in children, have not been well determined. We sought to establish an efficient enzyme-linked immunosorbent assay (ELISA) with a wide range of detection that would be suitable for measurement of serum TARC/CCL17 and to determine the normal ranges of this chemokine in different age groups and its diagnostic usefulness for AD. A sensitive specific ELISA for TARC/CCL17, which was previously reported, was modified to accommodate the wide range of TARC/CCL17 values often found in sera. Twenty-seven children with AD under 6 yr of age and 25 age-matched normal non-atopic controls, and 18 patients with AD and 27 controls who were 6 yr and older were enrolled. The severity of AD was evaluated using the SCORAD index. The serum levels of TARC/CCL17 were measured with the ELISA, and the serum levels of IP-10/CXCL10 were also measured. With the novel ELISA system, the assayable range of TARC/CCL17 was 14–8000 pg/ml, and the coefficient of variation at various concentrations ranged from 2.3% to 5.0%. The serum levels of TARC/CCL17 in normal individuals were significantly higher in young children, especially in the age group of 0–1 yr. The cut-off values of TARC/CCL17 for the diagnosis of AD were 1431 pg/ml for 0–1 yr group, 803 pg/ml for 2–5 yr group and 510 pg/ml for the 6 yr and older group, with high sensitivity and specificity of 0.83 and 0.93, 0.83 and 0.92, 0.85 and 0.96, respectively. The magnitude of the decrease in the SCORAD index after treatment with topical steroids correlated significantly with the decrease in serum TARC/CCL17. There was no difference in the serum levels of IP-10/CXCL10 between AD and the controls. The TARC/CCL17:IP-10/CXCL10 ratio tended to be higher in the control children aged 0–1 yr than in those aged 2–5 yr. The serum level of TARC/CCL17 reflects the severity and therapeutic response in AD. The high normal levels in infants should be taken into account when assaying TARC/CCL17.

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Key words: atopic dermatitis; infants; TARC/CCL17; IP-10/CXCL10; Th_1/Th_2 balance

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Thymus and activation-regulated chemokine (TARC)/CCL17 is a ligand for CC chemokine receptor 4 (CCR4) that is selectively expressed on Th_2 cells and may play an important role in the pathogenesis of allergic diseases such as atopic dermatitis (AD). TARC/CCL17 has been detected in the inflamed skin of an animal model of AD and in patients with AD and has been shown to recruit Th_2 cells into inflammatory sites (1, 2). The serum and plasma levels of this chemokine are also reported to be elevated in patients with AD, and the levels correlate closely with the disease severity, suggesting that TARC/CCL17 may be a good clinical marker for AD (3–5). To further characterize the clinical utility of TARC/CCL17, we previously showed that the serum level of TARC/CCL17 is 10- to 50-fold higher than its plasma level and that the serum/plasma ratio of TARC/CCL17 was significantly higher in AD patients than their normal counterparts (4). We also demonstrated that platelets contained and released TARC/CCL17 and that the TARC/CCL17 content in platelets from patients with AD was significantly higher than in those from normal individuals, suggesting that the serum level of TARC/CCL17 represents the circulating or plasma level of TARC/CCL17 plus additional protein released *ex vivo*, partly from activated platelets in AD. These observations indicate that the TARC/CCL17 level in serum may better reflect the disease severity of AD than its plasma level. In addition, as serum samples are easier to handle than plasma samples in ordinary laboratories, serum TARC/CCL17 would be a better clinical marker for AD.

In order to further establish the clinical utility of TARC/CCL17 measurement for diagnosis of AD, it is important to determine the normal range of TARC/CCL17. However, serum TARC/CCL17 levels in children, especially those at a young age, have not been well studied. It was reported that Th_2-skewed responses to common environmental allergens are seen in all newborns, suggesting default Th_2 deviation at a young age (6). This fact may have an impact on serum levels of Th_2-type chemokines such as TARC/CCL17.

Previously, we developed a sensitive enzyme-linked immunosorbent assay (ELISA) for TARC/CCL17 with a lower limit of detection of 1.4 pg/ml (7). Because the serum levels of TARC/CCL17 are very high, the assay has to be refined to be applicable to the range of TARC/CCL17 levels ordinarily found in serum from patients with AD. In this study, we have established a novel ELISA with a broad detection range for measurement of serum TARC/CCL17 and investigated the chemokine levels in normal children and patients with AD. We found that the serum TARC/CCL17 level was significantly elevated in normal infants compared with older subjects.

Table 1. Subjects

	Age (yr) group	n	Mean ± s.d.	Male /female
Atopic dermatitis	0–	27 (56)*	1.9 ± 1.8	17/10
	6 and above	13 (36)*	17.2 ± 8.2	9/9
Control	0–	25	2.0 ± 1.7	10/15
	6 and above	27	24.9 ± 11.5	11/8

*Total number of replicate measurements of TARC/CCL17.

Materials and methods

Subjects

Fifty-seven subjects were enrolled in the study. Diagnosis of AD was based on the published clinical criteria for the disease (8): an elevated serum immunoglobulin E (IgE) level and sensitivity to more than one inhalant or food allergen, demonstrated by a positive test for specific IgE antibodies performed with a UniCAP® system (Phadia, Uppsala, Sweden). As we focused on subjects of a young age, 27 patients with AD under 6 yr of age and 25 age-matched normal controls were included. Eighteen patients with AD and 27 controls who were 6 yr and over were also included (Table 1). In some patients with AD, blood samples for TARC/CCL17 were obtained more than two times during the course of treatment. Table 1 shows the total number of blood samples. The control subjects had no history of allergic diseases, with IgE serum levels of < 160 IU/ml for those aged 2 yr and above and 60 IU/ml for those < 2 yr old. They also had no detectable specific IgE antibodies to common inhalant and food allergens, namely, house dust mites, Japanese cedar pollen, cat dander, dog dander, egg white, milk and wheat. Subjects were recruited via the hospital web page and posters in the hospital. Many of the young volunteers, especially the infants, had an inguinal hernia and were scheduled for operation, but were otherwise healthy. Blood samples were drawn from these infants as part of the routine pre-operative blood testing. The severity of AD was evaluated using the SCORAD (severity scoring of atopic dermatitis) index (9). None of the patients were administered systemic corticosteroids.

This study was performed with the approval of the Ethics Committee of Mie National Hospital. Informed consent was obtained from all the subjects or the guardians of child subjects (< 16 yr old).

Blood sampling

Venous blood was drawn into vacuum tubes (Venoject II; Terumo, Tokyo, Japan) and allowed to stand for 1 h at room temperature. Then the serum was separated by centrifugation at 2000 ***g*** for 10 min. The obtained serum samples were stored below −20°C until chemokine assay.

ELISA for TARC/CCL17 (SD-8864)

Recombinant human TARC/CCL17 (hTARC/CCL17; PeproTech, Rocky Hill, NJ, USA) was used as a calibrator for the ELISA and an in-house antigen (7) was used for the experiment to test the assay precision. The in-house antigen was calibrated with the recombinant human TARC/CCL17, and both proteins had similar immunoreactivity in our ELISA (data not shown). Anti-hTARC/CCL17 monoclonal antibodies (mAb; 4A3 and 5F12) were purified from BALB/c mouse ascitic fluid. The preparation procedures for the in-house antigen and antibody have been described in detail elsewhere (7). The Fab' fragment of the 5F12 mAb was conjugated with horseradish peroxidase (HRP, EC 1.11.1.7; Boehringer Mannheim, Germany) as described elsewhere (10).

Each well of a 96-well microplate (Immuno Module F8 Maxisorp; Nunc, Roskilde, Denmark) was filled with a solution (200 µl) of anti-hTARC/CCL17 mAb 4A3 [10 mg/l in 10 mmol/l phosphate-buffered saline (PBS), pH 7.4] and incubated for 18 h at 4°C. After removal of the antibody solution, the wells were washed three times with 300 µl/well of PBS-T (PBS containing 0.5 ml/l Tween 20), followed by aspiration. PBS containing 10 g/l bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) and 100 g/l saccharose was added to each well of the microplate, which was then incubated for 1 h at 25°C. After aspiration, the microplate was dried in a desiccator under a vacuum for 4 h at 25°C and then stored at 4°C until use.

At the time of use, 100 µl of assay buffer (50 mmol/l phosphate buffer containing 0.3 mol/l NaCl, 0.5 ml/l Tween 20, 1 g/l BSA and 0.2 g/l NaN_3, pH 7.4) and then aliquots of hTARC/CCL17 standard (0–8000 pg/ml in assay buffer) or sample (25 µl each), were added to the wells of the immunoplate, which was then incubated for 2 h at 25°C. Each well was washed three times with PBS-T, and then 100 µl of Fab'-HRP (75 ng) in PBS containing 4 g/l BlockAce (Dainippon Pharmaceutical, Osaka, Japan) and 1 g/l Kathon CG (Rohm and Haas, Philadelphia, PA, USA) were added to each well, followed by incubation for 1 h at 25°C. The assay plates were washed five times with PBS-T, and then the immunoreactivity was visualized by addition of 100 µl/well of substrate solution (ABTS solution; Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at 25°C. The reaction was stopped by addition of 50 µl of 1 g/l sodium dodecyl sulphate to each well, and the absorbance was measured at 405 nm using an Immunoreader NJ-2000 (Nalge Nunc International K.K., Tokyo, Japan). The hTARC/CCL17 levels were calculated based on the standard curve for each assay plate, and experiments were performed in duplicate except when noted otherwise.

IP-10/CXCL10 assay

The serum levels of IP-10/CXCL10 were measured in subjects under 5 yr of age using a commercial kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The data on the serum levels of TARC/CCL17 and IP-10/CXCL10 were expressed as geometric means because the logarithmically transformed values of the data followed a normal distribution. Differences were analysed with the Mann–Whitney *U*-test for unpaired samples and with the Wilcoxon's signed rank test for paired samples. For multiple comparisons, ANOVA was followed by Dunnett's multiple comparison test. Spearman's correlation coefficients between two parameters were calculated. The discriminative usefulness of TARC/CCL17 was evaluated by constructing receiver operating characteristic(ROC) curves (11) where sensitivity vs. 1 − specificity was plotted for each possible cut-off level. For this analysis, the group with AD was labelled as diseased compared with the control group. The area under the curve was determined for each age subgroup. From each ROC curve, we determined the ideal cut-off level which corresponds to the closest point to the top left-hand corner and which most efficiently discriminates between the presence or absence of disease. The respective sensitivity, specificity and predictive values were calculated.

Results

Assay characteristics

A representative standard curve of this ELISA (SD-8864) is shown in Fig. 1. The assayable

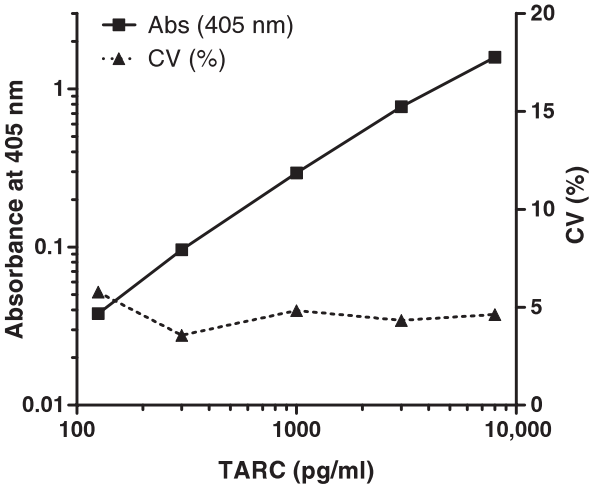

Fig. 1. A typical standard curve for the present ELISA for CCL17 and the precision profile (CV, %), calculated from five determinations for each point in one assay.

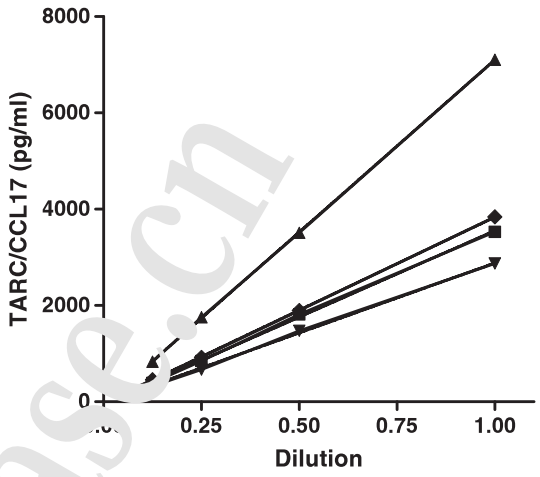

Fig. 2. Serial dilution linearity of the present ELISA. Four serum samples having different CCL17 concentrations were serially diluted with assay buffer.

range was 125–8000 pg/ml, and the coefficient of variation (CV) for the TARC/CCL17 concentration at each standard point ranged from 3.6% to 5.8% (n = 5). The reproducibility of this ELISA was estimated using clinically obtained sera having different TARC/CCL17 concentrations. The ranges of the intra- and inter-assay CVs were from 2.3% to 5.0% (n = 5) and 4.4% to 7.8% (n = 5), respectively, as shown in Table 2. Dilution curves of four sera showed preferable linearity (Fig. 2). The analytical recoveries were estimated for recombinant TARC/CCL17 added to serum samples containing three different concentrations of endogenous TARC/CCL17 (Table 3). The recovery ranged from 89.9% to 106.8%. Furthermore, we examined the cross-reactivity with other chemokines having similar structures and/or chemotactic activities for Th_2 cells leading to binding with the CC chemokine receptor 4 (CCR4) receptor. No cross-reactivity was found for TARC/CCL17 with 80 ng/ml preparations of the following chemokines: human MCP-1/CCL2 (PeproTech), eotaxin/CCL11 (12), MDC/CCL22 and mouse TARC/CCL17 (R&D Systems).

Table 2. Assay precision of the ELISA

	n	Mean (pg/ml)	s.d. (pg/ml)	CV (%)
Intra-assay				
Serum 1	5	263	13	5.0
Serum 2	5	1424	63	4.4
Serum 3	5	5817	133	2.3
Inter-assay				
Serum 4	5	239	11	4.8
Serum 5	5	844	37	4.4
Serum 6	5	3749	163	4.4

Table 3. Analytical recovery of standard CCL17 added to human serum

Sample	Endogenous (pg/ml)	Added (pg/ml)	Measured (pg/ml)	Found* (pg/ml)	Recovery (%)
Serum 7	214	500	694	480	96.1
	214	1000	1219	1006	100.6
	214	2000	2230	2017	100.8
Serum 8	1167	500	1701	534	106.8
	1167	1000	2167	1000	100
Serum 9	2883	500	3389	506	101.2
	2883	1000	3796	912	91.2
	2883	2000	4983	2100	105
Serum 10	3148	500	3664	516	103.2
	3148	1000	4047	899	89.9
	3148	2000	5138	1990	99.5

*Increase over endogenous CCL17.

Serum TARC/CCL17 levels in normal individuals

First, we examined the serum TARC/CCL17 level in normal non-atopic individuals of different ages. We found that the level in the age group of 0–5 yr was significantly higher than in the older age group of 6 yr and above ($p < 0.001$). We analysed that tendency by further dividing the subjects into four subgroups by age: < 2, 2–5, 6–15 and 16 yr and older. As shown in Fig. 3, the serum TARC/CCL17 level was significantly higher in the age group of 0–1 yr than in each of the other, older age groups (Fig. 3, geometric mean values are shown in the legend). As described in Materials and methods, the control subjects had normal serum IgE levels and were not sensitized to common inhalant and food allergens. In particular, the median serum IgE value in the age group of 0–1 yr was 6 IU/ml, and except for in one subject all IgE values were < 20 IU/ml. The serum TARC/CCL17 level of

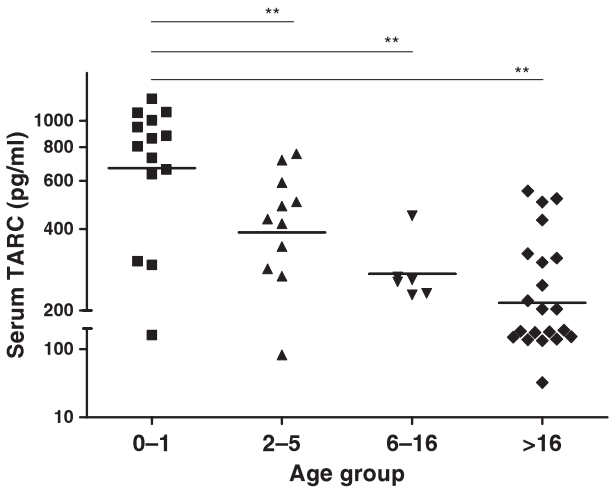

Fig. 3. Serum levels of TARC/CCL17 in normal individuals of various age groups. Horizontal lines indicate the geometric mean values, 707, 365, 273 and 213 pg/ml, in age groups of 0–1, 2–5, 6–16 and >16 yr, respectively. The serum TARC/CCL17 level was significantly higher in the age group of 0–1 yr than in the other age groups ($p < 0.01$; ANOVA and Bonferroni's multiple comparison test).

the subjects with the highest serum IgE, i.e. 56 IU/ml, was 512 pg/ml, which was below the median value in the group of 0–1 yr. There was no correlation between serum IgE and TARC/CCL17 in each group (data not shown).

Serum TARC/CCL17 levels in AD

Next, we compared the serum TARC/CCL17 level in AD with the normal controls in the corresponding age groups. In all age groups, the serum TARC/CCL17 level in AD was significantly elevated compared with the control subjects of corresponding age (Fig. 4; geometric mean values are shown in the legend). The values for the area under the ROC curves were 0.93, 0.89 and 0.96 in the respective age groups ($p < 0.001$) (Fig. 4). The cut-off values of TARC/CCL17 for the diagnosis of AD were 1431 pg/ml for the 0–1 yr old group, 803 pg/ml for the 2–5 yr old group and 510 pg/ml for 6 yr and older. With these cut-off values, the sensitivity and specificity of serum TARC/CCL17 for

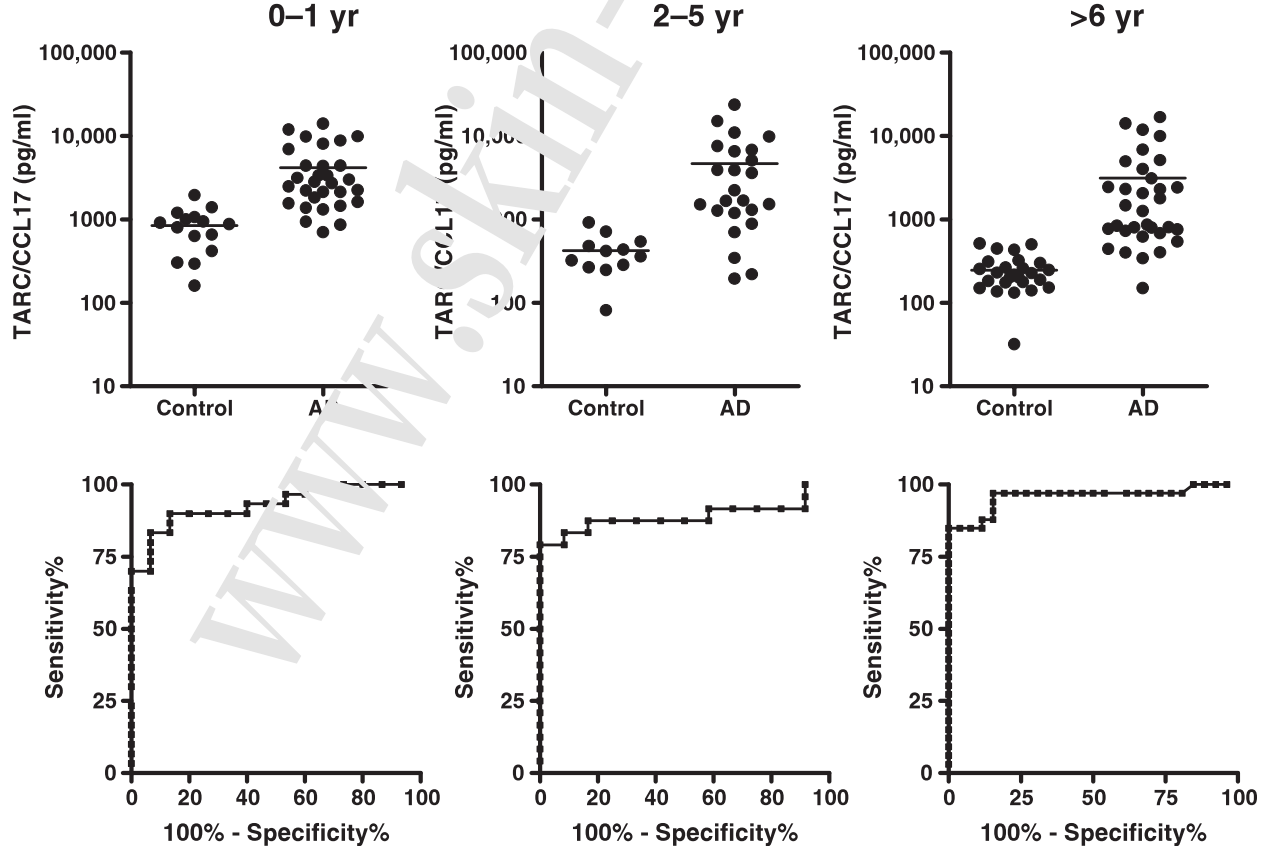

Fig. 4. Comparison of TARC/CCL17 levels between the controls and AD in age groups of 0–1, 2–5 and 6 yr and older (upper 3 graphs). The geometric mean values for the controls and AD in each age group were 707 and 3017, 365 and 2374 and 218 and 1550 pg/ml, respectively. The values for the area under the curve in ROC analysis (lower 3 graphs) in each age group were 0.931, 0.889 and 0.958, respectively.

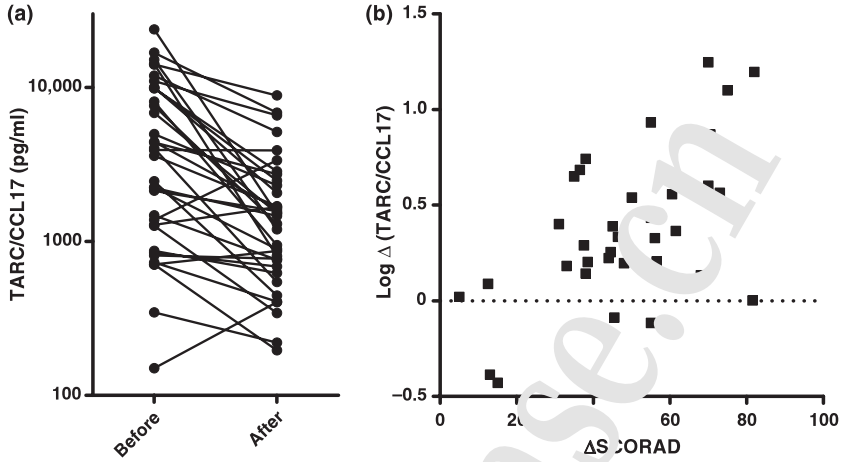

Fig. 5. (a) Changes in serum TARC/CCL17 before and after treatment. The geometric mean values (95% confidence interval) before and after treatment were 3170 (2061–4875) and 1293 (932–1793) pg/ml, respectively. (b) Relationship between decrease in TARC/CCL17 (log ΔTARC/CCL17) and that in SCORAD (ΔSCORAD) after treatment with topical steroids and emollients for patients with AD. The *Y*-axis shows the logarithmic transformation of the magnitude of decrease in the TACR/CCL17 level after treatment. The *X*-axis shows the magnitude of decrease in the SCORAD index after treatment. Spearman's correlation coefficient: 0.48; $p < 0.005$.

AD in the three age groups were 0.83 and 0.92, 0.83 and 0.92 and 0.85 and 0.96, respectively.

Relationship between serum TARC/CCL17 level and SCORAD index

We also found significant positive correlations between the serum TARC/CCL17 level and the severity of AD based on the SCORAD index in all age groups, with Spearman's correlation coefficients of 0.55, 0.65 and 0.61, respectively ($p < 0.01$). TARC/CCL17 was also measured in 35 patients before and after standard treatment with topical steroids, skin hydration and emollients. The serum levels of TARC/CCL17 decreased significantly after the treatment (Fig. 5a), in parallel with improvement in the symptoms. Analysis of the relationship between the change in the SCORAD index and the change in the serum TARC/CCL17 level after the treatment revealed that the magnitude of decrease in the SCORAD index correlated significantly with the decrease in the serum TARC/CCL17, with a Spearman's correlation coefficient of 0.48 ($p = 0.036$) (Fig. 5b). These results suggest that the serum TARC/CCL17 level reflects the disease activity of AD.

Serum IP-10/CXCL10 in normal infants

To address the question of whether a Th_2-biased immune status may be present in normal infants, the serum level of IP-10/CXCL10, a Th_1 chemokine, was measured in the two younger age groups, i.e. 0–1 and 2–5 yr. The IP-10/CXCL-10 level in AD was not elevated or decreased compared with in the age-matched control children, with levels of 200 and 185 pg/ml, respectively. In normal children, the CCL17:CXCL10 ratio tended to be elevated in the 0–1 yr group compared with the 2–4 yr old group ($p = 0.079$). There was no statistical difference in IP-10/CXCL10 between the two groups, although the geometric mean value was lower in the former group than in the latter: 186 vs. 286 pg/ml.

Discussion

The chemokines comprise a large protein family with a common property of inducing leucocyte chemotaxis. They are divided into four subfamilies, CXC, CC, CX3C and C, on the basis of structural motifs involving the first two N-terminal cysteine residues (13). Recently, a systematic nomenclature has been devised for chemokines and their corresponding 7-transmembrane G-protein-coupled receptors (14). Among them, TARC/CCL17 (15) is a ligand for CCR4, the shared receptor for MDC/CCL22 (16). CCR4 is expressed on Th_2 cells and a subset of cutaneous lymphocyte antigen $(CLA)^+$ skin-homing memory T cells (17, 18). CCL17 is produced by monocyte-derived dendritic cells, endothelial cells, bronchial epithelial cells, epidermal keratinocytes (13) and possibly platelets (4). Overproduction of CCL17 leading to Th_2 cell accumulation at the site(s) of inflammation is strongly implicated in the pathogenesis of allergic diseases such as AD and asthma (1, 19).

It has already been reported that the blood levels of a Th_2-type chemokine, TARC/CCL17, are significantly elevated in AD (3, 4, 20–23), which suggests that it might be possible to utilize TARC/CCL17 as a clinical marker of AD disease activity and responses to treatment. TARC/CCL17 measurement in clinical settings; however, requires development of an efficient assay system with sufficient sensitivity and range of detection for the diverse levels that may be found in clinical samples. A commercially available assay kit often used in earlier studies (20–23), that is, a sandwich enzyme immunoassay kit by R&D Systems, has the highest upper limit of detection, 2000 pg/ml. However, the serum TARC/CCL17 level in patients with AD often exceeds 2000 pg/ml (3, 4), and levels around 4000 pg/ml were reported in patients with severe AD (3). One may argue that dilution of samples to fit the assayable range would conserve precious samples from small children. However, one cannot know the proper dilution factor before assay, and in the end this approach may expend more sample by making it necessary to repeat the assay. In order to overcome this problem, we refined our existing sensitive ELISA system for TARC/CCL17 (7) so that it would have a broad range of detection from 25 to 8000 pg/ml. This range can accommodate the needs of clinical laboratories.

Another important issue for laboratory tests is elucidation of the normal ranges in different age brackets, especially in children, whose normal values usually differ from those in adults. Here, we found for the first time that the serum TARC/CCL17 levels were significantly higher in children younger than 6 yr of age, especially in those <2 yr old (Fig. 3). Based on this finding, we re-analysed the serum TARC/CCL17 level in AD in various age subgroups and confirmed that it was significantly elevated in all age strata (Fig. 4). To utilize the serum TARC/CCL17 level for evaluation of disease activity in AD, previous studies on this issue showed a positive correlation between CCL17 and severity indices such as SCORAD. However, this type of analysis can be confounded by the age factor as we have now found that TARC/CCL17 is elevated in infants. We also found a significant correlation between the TARC/CCL17 level and the SCORAD index in each age group. More importantly, to further exclude the possible influence of age, we analysed for correlation between the changes in the TARC/CCL17 level and the SCORAD index before and after treatment. We found that the decrease in TARC/CCL17 correlated significantly with the decrease in the SCORAD index after standard treatment with topical steroids and emollients (Fig. 5). These findings clearly demonstrate that the serum TARC/CCL17 level reflects the disease activity of AD in association with the response to treatment.

It is intriguing to speculate regarding the biological significance of the fact that TARC/CCL17, a Th_2 chemokine, is elevated in normal infants. A number of epidemiological studies have suggested that the increase in the prevalence of allergic disorders that has occurred over the past few decades in developed countries is attributable to reduced microbial burden during early childhood (24–27) [the so-called hygiene hypothesis (28)]. The immunological basis for the hygiene hypothesis is that reduced microbial stimuli in early childhood inhibits immune deviation from Th_2 to Th_1 immune responses (29) because various pathogen-associated molecular patterns such as CPG-containing oligodeoxynucleotides and lipopolysaccharide interact with toll-like receptors (TLRs) on antigen-presenting cells, including dendritic cells, to produce Th_1-polarizing interleukin-12. One of the present authors previously demonstrated that stimulation of murine Langerhans cells with *Staphylococcus aureus* Cowan 1, lipopolysaccharide and CpG, ligands for TLR2, 4 and 9, respectively, resulted in Th_1-polarizing cytokine production and inhibition of TARC/CCL17 (30). In this study, we also measured IP-10/CXCL10, a Th_1 attracting chemokine (13, 31), and found that the

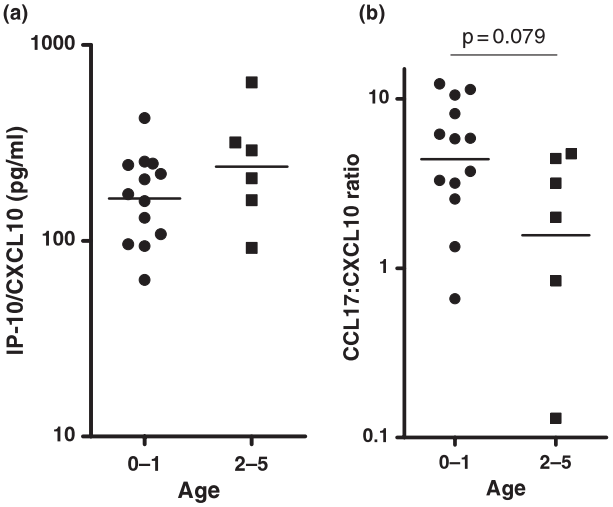

Fig. 6. (a) Serum levels of IP-10/CXCL10 in the control group 0–1 and 2–5 yr olds. The geometric mean values were 165 and 239 pg/ml, respectively. (b) Ratio of TARC/CCL17:IP-10/CXCL10 in the control group 0–1 and 2–5 yr olds. The geometric mean values were 4.4 and 1.6, respectively. p = 0.079 (Mann–Whitney *U*-test).

CCL17/CXCL10 ratio seemed to be elevated in the 0–1 yr age group compared with the 2–5 yr olds (Fig. 6). Overproduction of TARC/CCL17 in infancy might represent the default Th_2-polarized state, and lack of subsequent Th_1-polarizing stimuli might cause further production of the chemokine, leading to the development of AD and other atopic diseases. It was reported that neonatal BCG vaccination, which polarizes to Th_1, significantly reduced the asthma risk (32), and various attempts are currently being made to modify immune responses in order to prevent allergy. In this context, the normal levels of TARC/CCL17 may serve useful markers for monitoring the effects of immunoregulatory agents during treatment.

In summary, we have demonstrated that the normal serum level of TARC/CCL17 is significantly higher in early infancy. By taking into consideration the different normal ranges in different age groups, we re-established the clinical significance of serum TARC/CCL17 measurement in AD as a marker for the disease activity. Finally, an elevated normal level of TARC/CCL17 in infancy may represent a developmental aspect of the immune system and warrants further investigation of its role in the Th_1/Th_2 balance in health and allergy. Such studies may lead to development of immunomodulatory measures for prevention of allergic diseases in children.

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