

Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell-attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis

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Background: Tissue infiltration of CD4⁺ T cells plays an important role in the pathogenesis of allergic diseases. T-cell trafficking is mediated by specific chemokines and their receptors.

Objective: The purpose of this study was to investigate the participation of the chemokines thymus and activation-regulated chemokine (TARC) and cutaneous T cell-attracting chemokine (CTACK) in a large population of patients with allergic diseases.

Methods: Serum TARC and CTACK levels were measured in 455 patients with allergic disease. Patients were characterized as having atopic dermatitis (AD), allergic asthma, allergic rhinitis, or combinations or as healthy control subjects. Serum TARC and CTACK levels were correlated with disease activity in patients with AD. Furthermore, in 7 patients with AD, serum TARC and CTACK levels were studied after the start of systemic cyclosporin A treatment. Finally, TARC and CTACK localization was checked by immunohistochemistry in lesional skin biopsy specimens of patients with AD.

Results: Both TARC and CTACK serum levels in patients with AD were significantly higher than those in healthy control subjects and patients with allergic respiratory disease. Furthermore, serum TARC and CTACK levels significantly correlated with disease activity in patients with AD. Serum TARC levels paralleled clinical improvement in patients treated with cyclosporin A. Immunoreactive TARC was found in infiltrating cells and endothelial cells of the dermis but not in epidermal cells.

Conclusions: The serum TARC level is an objective parameter for disease severity specific for AD. Furthermore, it is a promising tool for treatment monitoring. (*J Allergy Clin Immunol* 2004;113:334-40.)

Key words: Allergic disease, asthma, atopic dermatitis, rhinitis, T_H2, chemokines, TARC, CTACK, cyclosporin A

Infiltration of inflammatory cells into tissues is regulated by chemokines. Chemokines are small, secreted polypeptides that regulate the tissue-specific recruitment and migration of lymphocytes by signaling through G protein-coupled 7-transmembrane receptors. Important chemokines in the skin-specific homing of T cells include thymus and activation-regulated chemokine (TARC) and cutaneous T cell-attracting chemokine (CTACK). The receptor for TARC is CCR4,¹ which is expressed mainly on skin homing, cutaneous lymphocyte antigen-positive (CLA⁺) T cells.² In vitro studies have shown that TARC induces the selective migration of lymphocytes of the T_H2 phenotype.^{1,3,4}

Infiltration of effector/memory CD4⁺ T cells of the T_H2 phenotype in the skin or the airways plays an important role in the pathogenesis of allergic diseases, such as atopic dermatitis (AD), allergic rhinitis (AR), and allergic asthma (AA). Kakinuma et al⁵ recently showed that serum TARC levels are elevated in patients with AD compared with patients with psoriasis and healthy control subjects (HCs). Furthermore, they showed that serum TARC levels correlate with the severity of AD.⁵

Various cell types, including endothelial cells,² dendritic cells,⁶ and keratinocytes,⁷ have been suggested to produce TARC. Furthermore, platelets have been found to contain large amounts of TARC,⁸ causing the large difference between plasma and serum TARC levels that have recently been reported.⁸

Studies showing TARC production in bronchial epithelial cell lines⁹ and nasal epithelial cells¹⁰ have suggested that TARC might also play a role in allergic respiratory diseases such as AA and AR.¹¹⁻¹⁵ However, clinical studies investigating serum levels in large groups of phenotypically well-defined patients with allergic diseases have not yet been performed.

CTACK is another recently identified chemokine that is thought to play an important role in the tissue-specific

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Received for publication September 12, 2003; revised November 24, 2003; accepted for publication December 3, 2003.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2003.12.007

Abbreviations used

AA: Allergic asthma
AD: Atopic dermatitis
AR: Allergic rhinitis
CLA: Cutaneous lymphocyte antigen
CsA: Cyclosporin A
CTACK: Cutaneous T cell-attracting chemokine
HC: Healthy control subject
LSS: Leicester Severity Score
PGA: Physician's Global Assessment
TARC: Thymus and activation-regulated chemokine

homing of T cells to the skin.¹⁶ CTACK is constitutively expressed in epidermal keratinocytes of the skin¹⁶ and is the ligand for CCR10. Similarly to TARC, CTACK preferentially attracts CLA⁺ T_H2 cells from peripheral blood.¹⁶⁻¹⁸ However, since CTACK is constitutively expressed by epidermal cells, it may be involved in basal trafficking of T cells through the skin during normal immune surveillance.¹⁷ Furthermore, in vitro experiments have demonstrated that CTACK production could be superinduced by pro-inflammatory cytokines, which, together with the increased expression of vascular E-selectin, may further explain the increase of memory T cells in the skin during cutaneous inflammation.¹⁸

The aim of this study was to investigate the specificity of increased TARC and CTACK serum levels in allergic disease and to evaluate TARC and CTACK as disease severity parameters in patients with AD. Finally, we studied the specific location of TARC and CTACK in lesional skin biopsy specimens of patients with AD by immunohistochemistry.

METHODS

Patients and samples

Sera from 455 human subjects were obtained (Table 1). Patients with AD were diagnosed according to the criteria of Hanifin and Rajka.¹⁹ Patients with AR were diagnosed on the personal history of allergic symptoms and a positive nasal provocation, as described by Lebel et al.²⁰ Patients with AA were selected on having a methacholine PC₂₀ of ≤9.8 mg/mL or a reversibility of ≥9% after inhalation of salbutamol.

Severity of AD was evaluated by using the Leicester Sign Score (LSS; range, 0 to 108), in which severity is scored by 6 clinical features (erythema, purulence, excoriation or crusting, dryness or scaling, cracking or fissuring, and lichenification) graded at 6 defined body sites on a scale of 0 (none) to 3 (severe).²¹ For all 455 sera, TARC levels were determined; from a subset of 163 samples, CTACK levels were determined.

In 7 patients with severe AD who had a flare, serum TARC and CTACK levels were determined before and during treatment with oral cyclosporine (CsA) (3 to 5 mg/kg per day). Severity of AD was evaluated by using the LSS, and response to treatment was scored by using the Physician's Global Assessment (PGA) score. The PGA is an overall assessment from 0 to 5 of a patient's eczema, taking into consideration the quality and extent of lesions relative to the baseline assessment (0 = clear [100%], 1 = almost clear [90% to 99% improvement], 2 = marked improvement [50% to 89%], 3 = modest improvement [<50%], 4 = no change, and 5 = worse).

TABLE 1. Demographics

Groups	N	Age (mean ± SD)	LSSsev (mean ± SD)
HC	31	30.0 ± 10.1	—
AA	16	29.6 ± 11.8	—
AA + AR	62	26.1 ± 12.4	—
AR	70	28.9 ± 10.0	—
AD	49	28.4 ± 11.4	23.00 ± 11.57
AD + AA	21	21.8 ± 10.2	18.90 ± 20.83
AD + AA + AR	120	26.3 ± 9.6	20.39 ± 12.15
AD + AR	86	27.0 ± 11.0	13.99 ± 6.52
Total	455	27.2 ± 10.8	—

ELISA

Serum TARC measurements were performed with the use of a flexible, 96-well polyvinyl chloride plate (Becton Dickinson Franklin Lakes, NJ) coated with a murine monoclonal antibody against human TARC (MAB364, R & D Systems, Abingdon, United Kingdom). A goat biotinylated TARC antibody was used for detection (BAF364, R & D Systems). The monoclonal antibody was applied to the plates overnight at 4°C. Plates were then washed 3 times with PBS containing 0.05% Tween-20 (PBS-Tween) and then blocked for 1 hour with 200 μL PBS containing 1% BSA per well at room temperature. Subsequently, standard dilutions and samples were added and incubated for 1 hour at room temperature. After washing as described above, plates were incubated for 1 hour with the biotinylated antibody. After washing as described above, the plates were incubated with streptavidin-poly horseradish peroxidase (Sanquin Research, Amsterdam, The Netherlands) diluted in PBS containing 2% milk (Protifar, Nutricia, Zoetermeer, The Netherlands) for 40 minutes at room temperature. After washing as described above, staining was performed by adding substrate (50-76-00, KPL, Gaithersburg, Md); the reaction was stopped by adding 1 mol/L phosphoric acid. Optical densities were measured at 450 nm with a Bio-Rad microplate reader (Biorad Laboratories, Inc, Hercules, Calif). Recombinant human TARC (364-DN, R & D Systems) was used as standard. The concentration of the chemokine was calculated from the standard curve generated by a curve-fitting program. The minimum detectable dose of TARC was 20 pg/mL.

Serum CTACK measurements were performed with the use of 96-well Costar EIA/RIA high-binding plates coated with a murine monoclonal antibody against human CTACK (MAB3761, R & D Systems). A biotinylated goat anti-human CTACK antibody (BAF376, R & D Systems) was used for detection. Recombinant human CTACK (376-CT, R & D Systems) was used as standard. The same protocol used for the TARC ELISA (described above) was used. The minimum detectable dose of CTACK was 20 pg/mL. Data for TARC and CTACK levels obtained are presented as means ± SDs.

Immunohistochemistry

Punch biopsy samples (3 mm) were taken from the (sub)acute lesional skin of patients with extrinsic AD (n = 5) and healthy control subjects (n = 3). The skin biopsy specimens were snap-frozen in liquid nitrogen, subsequently embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) OCT compound (Miles Inc), and stored at -80°C. Frozen 6-μm sections were cut and fixed in acetone. Samples were preincubated with normal goat serum (DAKO, Glostrup, Denmark) and subsequently incubated for 1 hour with rabbit anti-human TARC (Peprotech, Rocky Hill, NJ). The samples were then incubated for 60 minutes with biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, Calif). Samples were finally incubated for 30 minutes with streptavidin-AP (DAKO). After washing (PBS-Tween), samples were incubated for 30 minutes with avidin biotinylated alkaline phosphatase complex (DAKO). AP

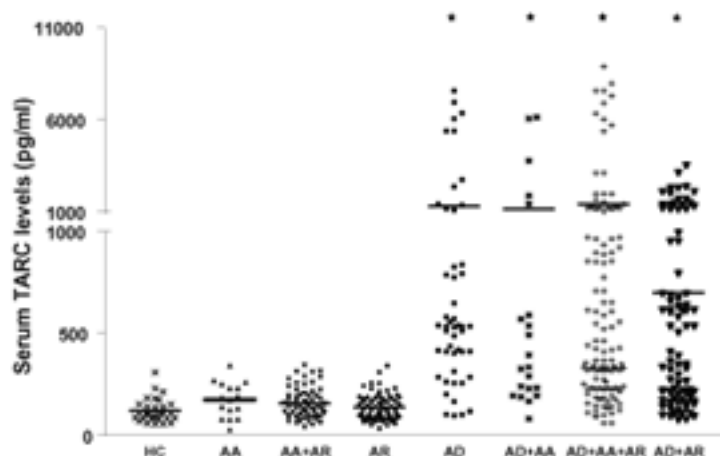


FIG 1. Serum levels of TARC in patients with AD are significantly increased ($*P < .05$) compared with HCs and patients with AA, AA + AR, and AR. Serum TARC levels are shown for patients with AA ($n = 16$), AA + AR ($n = 62$), AR ($n = 70$), AD ($n = 49$), AD + AA ($n = 21$), AD + AA + AR ($n = 120$), and AD + AR ($n = 86$) and for HCs ($n = 31$). Horizontal bars indicate mean values.

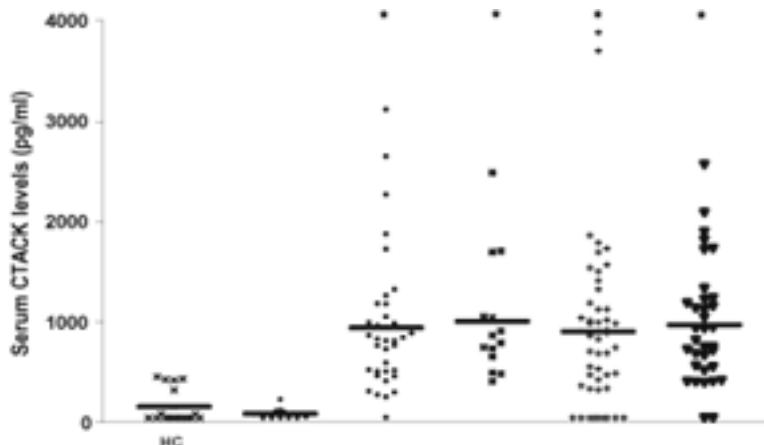


FIG 2. Serum levels of CTACK in patients with AD are significantly increased ($*P < .05$) compared with HC and patients with AR. Serum CTACK levels are shown for patients with AR ($n = 11$), AD ($n = 36$), AD + AA ($n = 14$), AD + AA + AR ($n = 49$), AD + AR ($n = 34$), and HC ($n = 19$). Horizontal bars indicate mean values.

activity was demonstrated for both chemokines by using naphthol AS-BI phosphate (Sigma, Steinham, Germany) as substrate and new fuchsin (Merck, Whitehouse Station, NJ) as chromogen until red staining was visible. Exogenous AP activity was inhibited by addition of levamisole (Sigma) to the reaction mixture. Samples were finally counterstained with Mayer hematoxylin.

Statistical analysis

Data were analyzed with the Mann-Whitney U test. Correlation coefficients were determined by using the Spearman rank correlation test. All comparisons were 2-sided. P values of $<.05$ were considered statistically significant.

RESULTS

Serum TARC and CTACK levels are specifically elevated in patients with AD

As shown in Figs 1 and 2, serum TARC and CTACK levels were increased in all 4 groups of patients with AD

with statistical significance ($P < .001$) compared with both nonallergic subjects (HCs) and patients with allergic respiratory disease only (AR, AA, and AA + AR).

Serum TARC and CTACK levels correlate with disease activity in AD

Serum TARC and CTACK levels correlated with LSS severity scores ($r = 0.56$ and 0.52 , respectively; $P < .001$) (Fig 3). No significant correlation was found between serum TARC levels and Lebel score in any of the patient groups with AD and AR. Furthermore, no significant correlation was found between the serum TARC levels and PC_{20} methacholine in any of the AD patient groups with AA (data not shown). The relatively low mean serum TARC value (699.2 ± 724 pg/mL) in the AD + AR group (Fig 1) can be explained by a lower mean LSS for that specific group of patients (13.9 vs 23.0, 18.9, and 20.4, respectively, in the other 3 AD-positive patient groups).

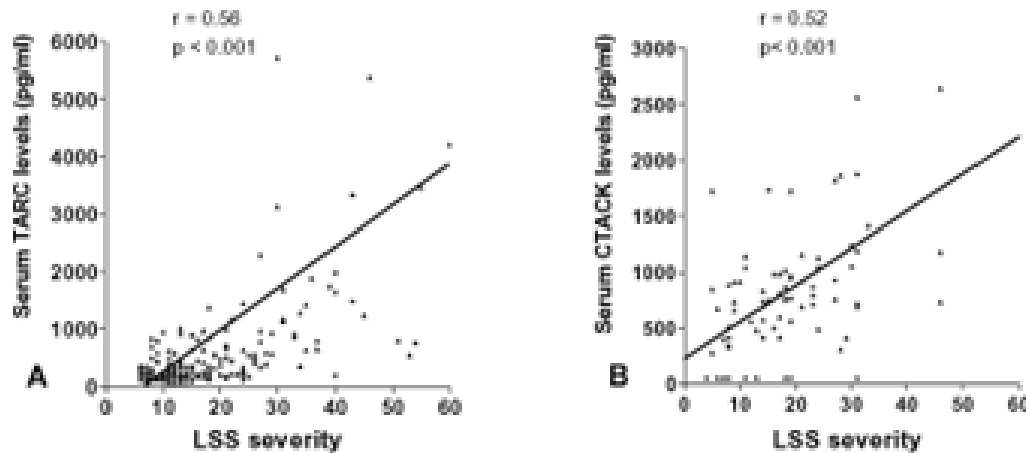


FIG 3. Serum TARC and CTACK levels in patients with AD correlated with disease severity. Comparison is shown between serum TARC levels ($n = 177$) and disease severity (LSS) (A) and between serum CTACK levels ($n = 76$) and disease severity (LSS) (B) in patients with AD.

Serum TARC levels during systemic treatment in patients with AD

In all 7 patients treated with systemic CsA, serum TARC levels showed a significant decline during the first month of treatment ($P < .01$). The average serum TARC level decreased from 5058 pg/mL before treatment to 643 pg/mL after 1 month of treatment (Fig 4). The serum TARC concentration decrease paralleled clinical improvement (all patients showed a PGA of ≤ 2 within 1 month of treatment). In 1 patient, serum TARC levels showed an increase during tapering of the dose of CsA. This increase was followed by clinical deterioration of eczema a few days later (data not shown).

Localization of TARC and CTACK in the skin

Immunoreactive TARC levels were detected in endothelial cells and dermal infiltrating cells in lesional skin of patients with AD (Fig 5) and to a much lesser extent in the skin of healthy control subjects (data not shown). No epidermal keratinocytes in lesional AD skin biopsy specimens or in HC skin biopsy specimens stained positive for TARC (Fig 5). CTACK was detected mainly in the basal layers of the epidermis in both AD and HC skin biopsy specimens (data not shown), as described previously.²²

DISCUSSION

To evaluate a possible role for serum chemokine levels in atopic diseases, we determined serum TARC and CTACK levels in phenotypically well-defined groups of allergic patients. We conclude that serum TARC levels are specifically elevated in patients with AD irrespective of allergic respiratory comorbidity. Several recent reports have suggested that TARC might be important not only in allergic skin disease but also in allergic respiratory diseases (AA and AR).^{11,13} The discrepancy between our results and other studies that have investigated patients with allergic respiratory disease^{11,12} may be the result of

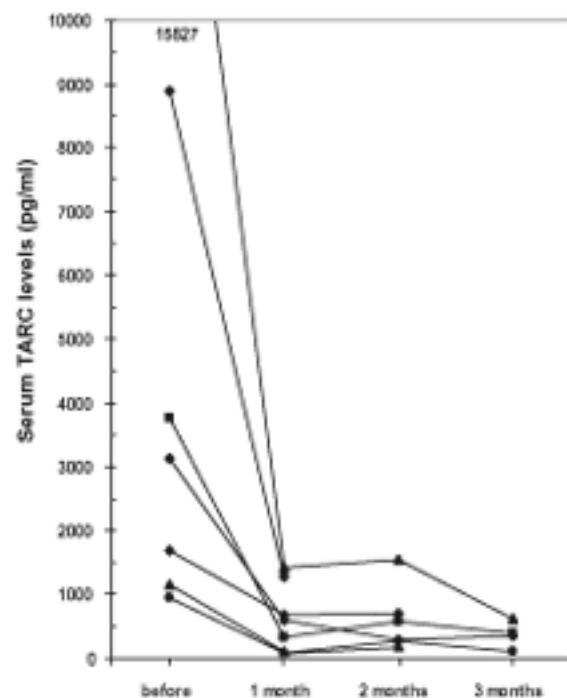


FIG 4. Serum TARC levels show significant decrease during treatment with cyclosporine. Serum TARC level of 7 patients with AD with a flare were evaluated before and during treatment with cyclosporin A. Four patients were given cyclosporine at a dose of 5 mg/kg body weight per day; 2 patients received a dose of 3 mg/kg per day for the duration of the study. All patients showed a 50% to 99% improvement after 1 month of treatment. Serum CTACK levels showed a less prominent decrease (from 1438.4 pg/mL before treatment to 832.6 pg/mL after 1 month and 722.5 pg/mL after 2 months) compared with serum TARC levels.

the inclusion of patients with AD in the allergic respiratory disease patient groups. However, several findings have indicated that bronchial epithelial cells and nasal epithelial cells can produce TARC.^{10,14,23} In patients with AR, low serum TARC levels might be explained by

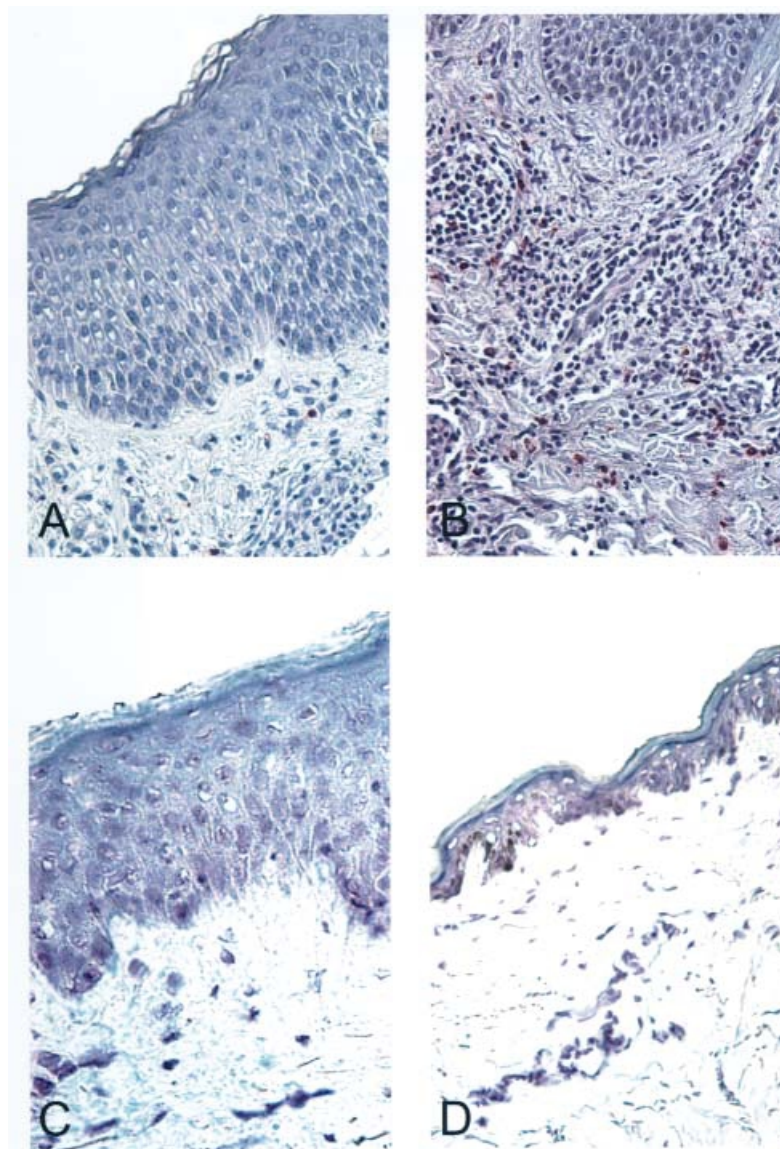


FIG 5. Immunoreactive TARC was detected in the lesional skin of patients with AD. **A**, No positive staining was detected in the epidermis. **B**, TARC was detected in the dermal endothelial cells and infiltrating dermal cells of the lesional AD skin. **C**, Isotype control. **D**, No immunoreactive TARC was found in biopsy specimens from skin of HC (original magnification $\times 200$).

local production of TARC in nose epithelial cells, being insufficient to lead to a systemic increase. However, in patients with AA, despite the large epithelial surface, we also did not find increased serum TARC levels. This indicates that the results of studies that use bronchial cell lines and mouse models cannot simply be extrapolated to normal human airway cells *in vivo*.

A large variety of laboratory²⁴ and clinical measurements²⁵ have been linked to disease severity in AD, which reflects the difficulties in defining a selective and objective marker that best relates to disease severity. This has led to a wide variation in outcome measurements in clinical studies that is hindering evidence-based practice.²⁶ The chemokines TARC and CTACK have recently been suggested as useful objective mark-

ers for disease activity in AD.^{5,22} In this report, we have additionally shown that TARC and CTACK serum levels correlate with LSS severity and are specific markers for AD. This substantiates the usefulness of these parameters as objective markers for disease severity in AD. Furthermore, we have shown that in patients with severe AD, serum TARC levels parallel disease severity during treatment with CsA. Moreover, the data indicate that TARC may play a role early in the cascade leading to skin inflammation, making it a possible target for therapy. CTACK serum levels were also tested during CsA treatment; however, we found a less pronounced correlation with disease severity in patients treated with CsA in comparison with TARC. This may be explained by the more constitutive production of CTACK.¹⁷

We have measured TARC levels in serum. Fujisawa et al⁸ have recently shown that platelets contain TARC and that furthermore the TARC content of platelets in patients with AD is dramatically elevated. We determined serum/plasma TARC ratios for 10 patients with AD and 10 HCs (with comparable platelet counts) and also found an increased serum/plasma TARC ratio in patients with AD (average $\pm$ SD, 6.2 ± 2.0) compared with HCs (average $\pm$ SD, 2.4 ± 0.8). The role of elevated TARC levels localized in platelets of patients with AD remains unknown but might be explained by the phenomenon of chemokine sink, as described in erythrocytes.²⁷

The cellular source of TARC production has been the subject of debate since it was first described in 1996.²⁸ Early reports showed immunohistochemical staining for TARC localized to the endothelial and infiltrating cells in AD skin biopsy specimens^{2,29} and production primarily by monocytes and dendritic cells.^{6,30} However, other publications showed immunohistochemical staining for TARC in the epidermis and no staining in the vessels of the skin.^{5,31} These latter findings were substantiated by in vitro experiments with the transformed keratinocyte cell line HaCaT³²⁻³⁴ and in a mouse model for AD that also showed immunohistochemical staining for TARC in the epidermis.³⁵ However, Albanesi et al³⁶ and Lebre et al (manuscript submitted) failed to induce TARC production in primary cultures of human keratinocytes. We found positive staining for TARC in endothelial cells and infiltrating cells in the dermis of lesional AD skin biopsy specimens. No immunoreactive TARC was found in the epidermis of AD lesional skin biopsy specimens. Our results further substantiate the suggestion that production of TARC is a phenomenon specific to the HaCaT cell line and is not relevant for normal human keratinocytes.³⁷ CTACK was found to be expressed at high levels in the epidermis of lesional skin biopsy specimens from patients with AD compared with nonlesional skin and skin biopsy specimens from HCs. Taken together, these findings support the hypothesis that TARC and CTACK may work in a sequential way, with TARC acting mainly in the first steps of T-cell recruitment by inducing integrin-dependent adhesion and transendothelial migration of T cells⁴ and CTACK playing a major role in the migration of T cells into the upper layers of the skin. Finally, T cells infiltrating the epidermis may result in the induction of apoptosis in keratinocytes, resulting in spongiosis, the histologic hallmark of eczema.³⁸

In conclusion, elevated serum TARC levels are specific for patients with AD. Additionally, we have shown that serum TARC but not CTACK levels correlate with disease severity during systemic treatment with CsA. In summary, we suggest that the use of TARC as a selective, objective, and quantitative outcome measure will eventually lead to better interpretability of study results in the future. Further research will determine the potential roles of TARC as an objective and possibly predictive marker in the management and monitoring of AD.

We thank the DUMAS group³⁹ and Marja Oldhoff for collecting the patient serum samples. We also thank Inge de Vegt and Marloes Laaper for excellent technical support.

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