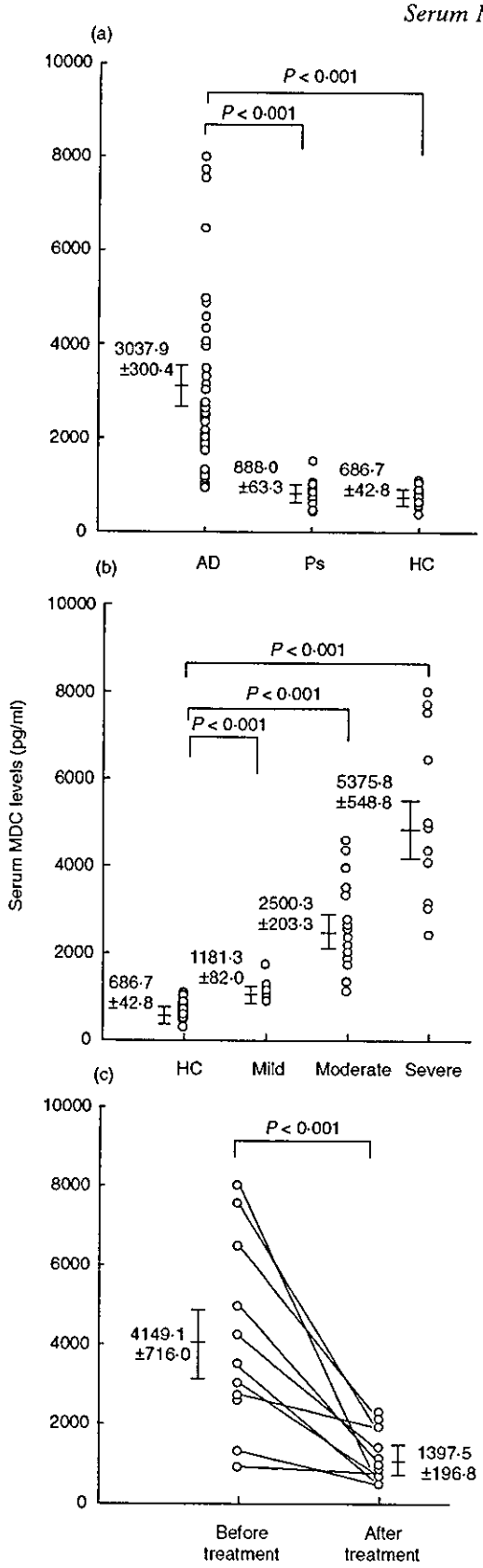

serum soluble interleukin-2 receptor (sIL-2R), serum TARC levels and eosinophil numbers in peripheral blood.

MATERIALS AND METHODS

Samples and reagents

Sera of 45 AD patients clinically diagnosed according to the criteria by Hanifin and Rajka [1], 25 psoriasis vulgaris patients and 25 healthy controls were examined. The 45 AD patients were treated with topical corticosteroid therapy in combination with oral antihistamine drugs. Twenty of the 45 patients had a personal history of respiratory allergy, whereas the patients with psoriasis vulgaris and the healthy controls had no allergies and their total serum IgE levels were within the normal range. After venous blood was drawn, serum was separated and stored at −20°C until use.

The disease severity of AD was determined by the modified SCORAD (SCORing Atopic Dermatitis) index system [15,16]. In this study, we also divided the AD patients into three groups, mild, moderate and severe, according to the proposal for severity grading of AD using only objective criteria [16].

ELISA

The serum levels of MDC, sIL-2R, sE-selectin and TARC were measured using an ELISA system. A 96-well microplate coated with either murine MoAb against human MDC (R & D Systems Inc., Mineapolis, MN, USA), sIL-2R (R & D Systems Inc.), sE-selectin (MedSystems, Vienna, Austria) or TARC (Techne Corp., Mineapolis, MN, USA) was used. Each serum was added to the microplate and incubated. After the removal of unbound material by aspiration and washing, a conjugate of MDC, sIL-2R, sE-selectin or TARC was detected by its reaction with a substrate solution, which yielded a coloured product proportional to the amount of conjugate. The optical density of each well was measured at 450 nm.

Statistical analysis

The data were analysed using the Mann–Whitney *U*-test. A P value $< 0{\cdot}05$ was considered to be statistically significant.

Correlation coefficients were determined using the Spearman rank correlation test. All comparisons were made after logarithmic transformation. Statistical analysis was also calculated and a P value $< 0{\cdot}05$ was considered to be statistically significant.

RESULTS

Serum MDC levels in AD patients were significantly higher than those in psoriasis vulgaris patients or healthy controls

The average level of serum MDC in the AD patients was 3037·9 ± 300·4 pg/ml. This elevation was statistically significant

Fig. 1. Serum MDC levels in AD patients, healthy controls and psoriasis patients. (a) Serum MDC levels in the AD patients ($n = 45$) were significantly higher than those in the psoriasis vulgaris patients (Ps) ($n = 25$) or healthy controls ($n = 25$). (b) Serum MDC levels among healthy controls and three groups of AD patients: mild, moderate and severe. Serum MDC levels in the severe group ($n = 13$) were significantly higher than those in the mild ($n = 10$) or moderate ($n = 22$) groups. (c) Serum MDC levels were measured before and after treatment with topical corticosteroid ($n = 11$). Serum MDC levels significantly decreased after treatment.

compared with that in psoriasis vulgaris patients (888·0 ± 63·3 pg/ml, $P < 0·001$) or healthy controls (686·7 ± 42·8 pg/ml, $P < 0·001$) (Fig. 1a). Thus, the value of serum MDC in AD patients was remarkably higher than that in psoriasis vulgaris patients and healthy controls.

Next, we divided the 45 AD patients into three groups, mild ($n = 10$), moderate ($n = 22$) and severe ($n = 13$), and compared the serum MDC levels among these different groups. The serum MDC levels of the severe AD group (5375·8 ± 548·8 pg/ml) were significantly higher than those of the mild AD group (1181·3 ± 82·0 pg/ml, $P < 0·001$) or the moderate AD group (2500·3 ± 203·3 pg/ml, $P < 0·001$) (Fig. 1b).

Serum MDC levels decreased after treatment with topical corticosteroid treatment and antihistamine drugs

We measured serum MDC levels in 11 patients with AD before and after treatment. The serum MDC levels of the AD patients were high before treatment (4149·1 ± 716·0 pg/ml, SCORAD 60·2 ± 15·3) and significantly decreased after treatment in accordance with the improvement of the eruption (1397·5 ± 196·8 pg/ml, SCORAD 29·6 ± 10·0, $P < 0·001$) (Fig. 1c). Serum sE-selectin, sIL-2R levels and eosinophil numbers in peripheral blood significantly decreased in parallel with the changes in serum MDC levels during treatment (data not shown).

Serum MDC levels correlated with disease activity in AD patients

The serum MDC levels of AD patients were compared with serum sE-selectin, sIL-2R, IgE levels, eosinophil numbers in peripheral blood and SCORAD. The serum sE-selectin and sIL-2R levels of AD patients were significantly higher than those in the healthy controls (data not shown), which is consistent with previous reports [5–7]. The serum MDC levels significantly correlated with SCORAD ($r = 0·68$) (Fig. 2a), serum sE-selectin levels ($r = 0·66$), serum sIL-2R levels ($r = 0·65$), serum TARC levels ($r = 0·72$) (Fig. 2b) and eosinophil numbers in peripheral blood ($r = 0·74$).

DISCUSSION

In this study, we examined the relevance of MDC in AD and obtained results as follows: (i) serum MDC levels in AD patients were significantly higher than those in psoriasis vulgaris patients or healthy controls; (ii) serum MDC levels in the severe group of AD patients were significantly higher than those in the mild or moderate groups; (iii) serum MDC levels in AD patients decreased after treatment in accordance with the improvement of the eruption; and (iv) serum MDC levels in AD patients significantly correlated with SCORAD, serum sE-selectin levels, serum sIL-2R levels, serum TARC levels and eosinophil numbers in peripheral blood.

MDC (CCL22) is a member of the CC chemokine family and one of the ligands for CCR4 [10]. It is constitutively expressed in DCs [9], macrophages [9] and thymic epithelial cells [17], and acts as a chemoattractant for CCR4-expressing cells such as memory T cells. It is also a chemoattractant for NK cells and eosinophils, even though they have little or no CCR4 expression [9,18].

Previous *in vitro* studies revealed that MDC production from monocytes is stimulated by IL-4 and IL-13, whereas it is inhibited by IFN-γ [19,20]. Moreover, MDC production from activated T cells is found to be preferentially associated with a Th2-type cytokine profile and inversely related to IFN-γ production *in vitro* [14,21]. From these data, it is suggested that MDC expression is preferentially involved in Th2-type reactions.

Previously, it was reported that MDC production is up-regulated at the mRNA and protein level during an allergic reaction in mouse airway hyperreactivity and lung inflammation [22]. In addition, we have clarified that in NC/Nga mice, regarded as a mouse model for human AD, MDC is observed in lesional and, to a lesser extent, non-lesional skin [12]. Thus, it is suggested that

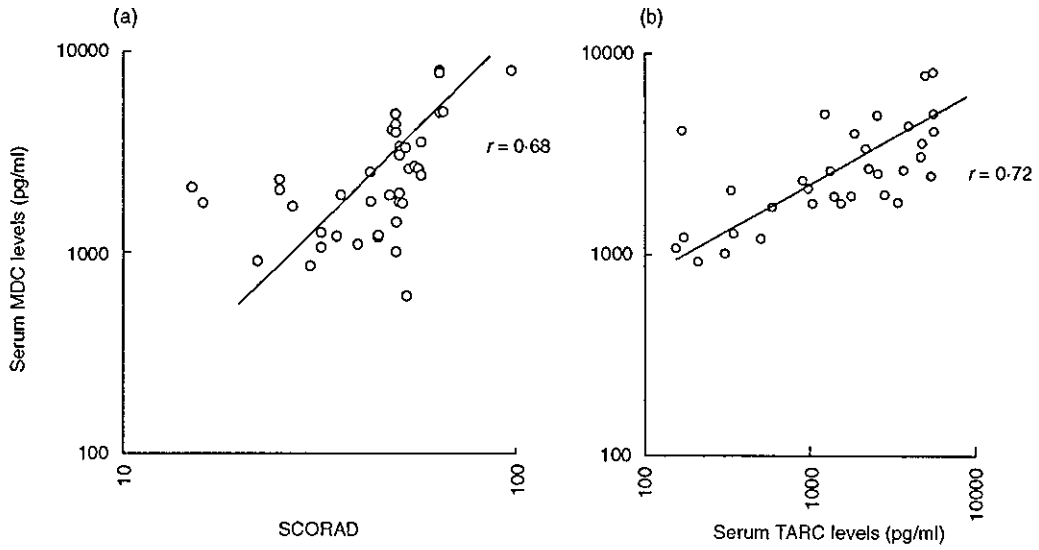

Fig. 2. Comparison between serum MDC levels and SCORAD (a) and serum TARC levels (b) in AD patients. Serum MDC levels in AD patients correlated with SCORAD ($P < 0·001$) (a) and serum TARC levels ($P < 0·001$) (b).

MDC may be involved in allergic diseases. Indeed, a recent study revealed increased serum MDC levels in patients with AD and immunoreactive MDC in the lesional skin [14]. These results strongly suggest the participation of MDC in diseases involving infiltration of predominantly Th2-type cells, although little has been elucidated about the precise function of MDC in AD. In this report, we confirmed that the serum MDC levels of AD patients were significantly higher than those of psoriasis vulgaris patients or healthy controls; we demonstrated that the serum MDC levels in AD were higher in the severe group than in the moderate or mild groups, and that these high levels decreased after treatment in accordance with the improvement of the eruption in the AD patients. In addition, we demonstrated the correlation of serum MDC levels with other laboratory data such as serum sIL-2R, sE-selectin and TARC levels, and eosinophil numbers in peripheral blood, which are reported to be disease markers for AD [5–7,13]. The serum MDC levels significantly correlated with SCORAD, a measure of clinical severity of AD. Thus, these data clearly show that serum MDC levels may be an important marker for the disease activity of AD.

It was recently reported that immunoreactive MDC is observed in $CD3^+$ T lymphocytes and $CD1a^+$ DCs in the lesional skin of AD patients [14], whereas we have reported that TARC, another ligand for CCR4, is highly expressed in the lesional keratinocytes of AD patients [13]. Thus, TARC and MDC are differentially expressed in the lesional skin of AD, although both chemokines attract CCR4-expressing cells.

In conclusion, we have confirmed that serum MDC levels clearly reflect the disease activity of AD. From these data, we strongly suggest that MDC, as well as TARC, may be involved in the pathogenesis of AD.

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Significant elevation of serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, in patients with atopic dermatitis: serum eotaxin-3/CCL26 levels reflect the disease activity of atopic dermatitis

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SUMMARY

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterized by the predominant infiltration of T cells, eosinophils and macrophages in lesional skin. Recently, eotaxin-2/CCL24 and eotaxin-3/CCL26 were identified as CC chemokines that signal exclusively via the CCR3 receptor and have eosinophil-selective chemoattractant activity, as does eotaxin/CCL11. We previously reported that serum levels of thymus and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22 were correlated with the severity of AD. In this report, we investigated the participation of eotaxin-2/CCL24 and eotaxin-3/CCL26 in AD, first measuring the serum levels of eotaxin-2/CCL24 and eotaxin-3/CCL26 in 30 patients with AD, 20 patients with psoriasis vulgaris and 20 healthy controls. The serum levels of eotaxin-3/CCL26 (but not eotaxin-2/CCL24) were significantly higher in patients with AD than in either healthy controls or patients with psoriasis vulgaris; furthermore, the eotaxin-3/CCL26 levels in patients with moderate and severe AD were significantly higher than eotaxin-3/CCL26 levels in patients with mild AD. The serum eotaxin-3/CCL26 levels tended to decrease after treatment, but there was no significant difference between groups. Moreover, the serum eotaxin-3/CCL26 levels were significantly correlated with the serum TARC/CCL17 and MDC/CCL22 levels, eosinophil numbers in peripheral blood and the scoring AD (SCORAD) index. Our study strongly suggests that serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, have a notable correlation with disease activity of AD and that eotaxin-3/CCL26, as well as TARC/CCL17 and MDC/CCL22, may be involved in the pathogenesis of AD.

Keywords eotaxin-2/ CCL24 eotaxin-3/ CCL26 atopic dermatitis disease activity

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disease that is characterized by pruritic and eczematous lesions which chronically persist. It is often associated with elevated serum immunoglobulin E (IgE) levels and peripheral blood eosinophilia. Histopathologically, the skin lesions in AD show infiltration of eosinophils, T lymphocytes and macrophages [1,2]. We previously showed that serum thymus and activation-regulated chemokine (TARC/CCL17) levels and serum macrophage-derived chemokine (MDC/CCL22) levels are significantly correlated with the disease activity of AD, as well as with eosinophil numbers in peripheral blood [3,4].

Both eotaxin-2/CCL24 and eotaxin-3/CCL26 are CC chemokines and functional ligands for CC chemokine receptor 3 (CCR3), which is preferentially expressed on eosinophils. After identification of eotaxin/CCL11 as a relatively selective chemokine for eosinophils [5,6], eotaxin-2/CCL24 was cloned and showed chemotactic activity for eosinophils in a range similar to that of eotaxin/CCL11 [7,8]. More recently, a new member of the eotaxin family – eotaxin-3/CCL26 – has been cloned by two different research groups [9,10]. Only 34% of the amino acids in eotaxin-2/CCL24 and eotaxin-3/CCL26 are identical [10], but these two chemokines have a common characteristic feature in terms of chemotaxis for eosinophils via the CCR3 [7–11]. However, the precise role of these chemokines in AD remained unclear.

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To clarify the relationship between AD, eotaxin-2/CCL24 and eotaxin-3/CCL26, we measured the serum levels of eotaxin-2/CCL24 and eotaxin-3/CCL26 and compared them in patients with AD, patients with psoriasis vulgaris and healthy controls. We also compared them with the clinical and laboratory data that reflected the disease activity of AD.

SUBJECTS AND METHODS

Subjects

Thirty patients [mean age ± standard deviation (s.d.): 28·7 ± 7·1 years] with AD, 20 patients (28·2 ± 6·1 years) with psoriasis vulgaris, and 20 control subjects (30·4 ± 10·3 years) were enrolled in this study. All patients with AD were given diagnoses according to the criteria of Hanifin & Rajka [1], and were treated with topical corticosteroids in combination with oral antihistamines. Disease activity of AD was determined by the modified scoring atopic dermatitis (SCORAD) index [12,13]. We divided the patients with AD into three categories, based on severity of the condition, using only objective criteria of SCORAD (mild, < 15; moderate, 15–40; severe, > 40) [13]. In eight patients with AD, we collected the sera before and after treatment. The 20 healthy controls had no history of allergy or psoriasis. All sera were stored at –20°C until use.

Enzyme-linked immunosorbent assay (ELISA)

TARC/CCL17 and MDC/CCL22 immunoassay kits were obtained from Genzyme TECHNE (Minneapolis, MN). Eotaxin-2/CCL24 and eotaxin-3/CCL26 immunoassay kits were from R & D systems (Minneapolis, MN). Serum levels of eotaxin-2/CCL24, eotaxin-3/CCL26, TARC/CCL17 and MDC/CCL22 were measured according to the manufacturer's instructions. Optical densities were measured at 450 nm using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The concentrations were calculated from the standard curve generated by a curve-fitting program. Data obtained from the ELISA are presented as mean ± s.d.

Statistical analysis

Data were analysed using the Mann–Whitney *U*-test. A *P*-value of <0·05 was considered to be statistically significant. Correlation coefficients were determined by using the Spearman rank correlation test. Statistical analysis was also performed, and a *P*-value of <0·05 was considered statistically significant.

RESULTS

Serum eotaxin-2/CCL24 and eotaxin-3/CCL26 levels in patients with AD, patients with psoriasis vulgaris and healthy controls

The mean serum eotaxin-2/CCL24 levels of patients with AD, patients with psoriasis vulgaris and control subjects were 179·8 ± 114·2 pg/ml, 246·4 ± 178·2 pg/ml, and 223·1 ± 128·6 pg/ml, respectively; there was no significant difference between these groups (Fig. 1a). In contrast, the mean serum level of eotaxin-3/CCL26 of patients with AD was 46·1 ± 20·5 pg/ml, which was significantly higher than that of patients with psoriasis vulgaris (24·2 ± 4·7 pg/ml, $P < 0·001$), or controls (34·1 ± 15·3 pg/ml, $P < 0·01$) (Fig. 1b). In AD patients, in the categories of mild, moderate and severe, the serum eotaxin-3/CCL26 levels were 27·6 ± 3·1 pg/ml, 48·8 ± 16·1 pg/ml and 62·2 ± 24·2 pg/ml, respectively (Fig. 1c). The levels in the groups with moderate and severe AD were significantly higher than those in the group with mild AD ($P < 0·001$, $P < 0·005$, respectively).

Serum eotaxin-2/CCL24 and eotaxin-3/CCL26 levels in patients with AD, before and after treatment

In eight patients with AD, we evaluated serum eotaxin-2/CCL24 and eotaxin-3/CCL26 levels before and after topical corticosteroid treatment in combination with oral antihistamines. The serum eotaxin-2/CCL24 levels decreased from 343·0 ± 182·6 pg/ml to 194·8 ± 112·5 pg/ml after the treatment (Fig. 2a) and the serum eotaxin-3/CCL26 levels decreased from 42·6 ± 18·2 pg/ml to 32·8 ± 13·5 pg/ml (Fig. 2b). However, there was no significant difference between these levels.

Correlation between serum eotaxin-2/CCL24 and eotaxin-3/CCL26 levels and other clinical or laboratory data

Because the serum eotaxin-3/CCL26 levels in patients with AD were significantly higher than those in patients with psoriasis vulgaris and in healthy controls, we next compared them with other clinical or laboratory data: serum TARC/CCL17 and MDC/CCL22 levels; eosinophil numbers in peripheral blood; and SCORAD. The serum eotaxin-3/CCL26 levels correlated significantly with the serum TARC/CCL17 levels ($r = 0·50$, $P < 0·05$), the serum MDC/CCL22 levels ($r = 0·46$, $P < 0·05$), eosinophil numbers in peripheral blood ($r = 0·44$, $P < 0·05$) and SCORAD ($r = 0·55$, $P < 0·01$) (Table 1). In addition, the serum TARC/CCL17 and MDC/CCL22 levels were significantly correlated with the SCORAD, eosinophil numbers in peripheral blood and each other, consistent with our previous reports (data not shown) [3,4]. In contrast, serum eotaxin-2/CCL24 levels were not correlated with these clinical or laboratory data (data not shown).

DISCUSSION

Eosinophils are involved in allergic diseases, such as asthma, rhinitis and AD [14]. Elucidation of the mechanisms underlying the accumulation of eosinophils in inflamed tissues is of critical importance for understanding the onset and progress of these eosinophilic diseases. Previous studies reported that the serum eotaxin/CCL11 levels were significantly correlated with the disease activity of AD [15] and that immunoreactivity and transcripts of eotaxin/CCL11 and CCR3 were significantly increased in lesional skin from AD [16]. Furthermore, a previous study reported that rhinovirus infection up-regulated eotaxin-2/CCL24 expression in bronchial epithelial cells [17]. However, little is known about the relationship between eotaxin-2/CCL24, eotaxin-3/CCL26 and AD.

In this study, we clearly showed that the serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, in patients with AD were significantly higher than those in patients with psoriasis vulgaris and healthy controls, although the serum level of eotaxin-3/CCL26 was lower than that of eotaxin-2/CCL24. Moreover, the serum eotaxin-3/CCL26 levels significantly increased in accordance with the clinical severity of AD. However, there was no significant difference before and after treatment for AD, although the elevated serum eotaxin-3/CCL26 levels tended to decrease after the treatment.

To clarify the relationship between eotaxin-3/CCL26 and AD in detail, we next compared the serum eotaxin-3/CCL26 levels with the disease activity of AD and found that these levels are significantly correlated with SCORAD, the serum TARC/CCL17

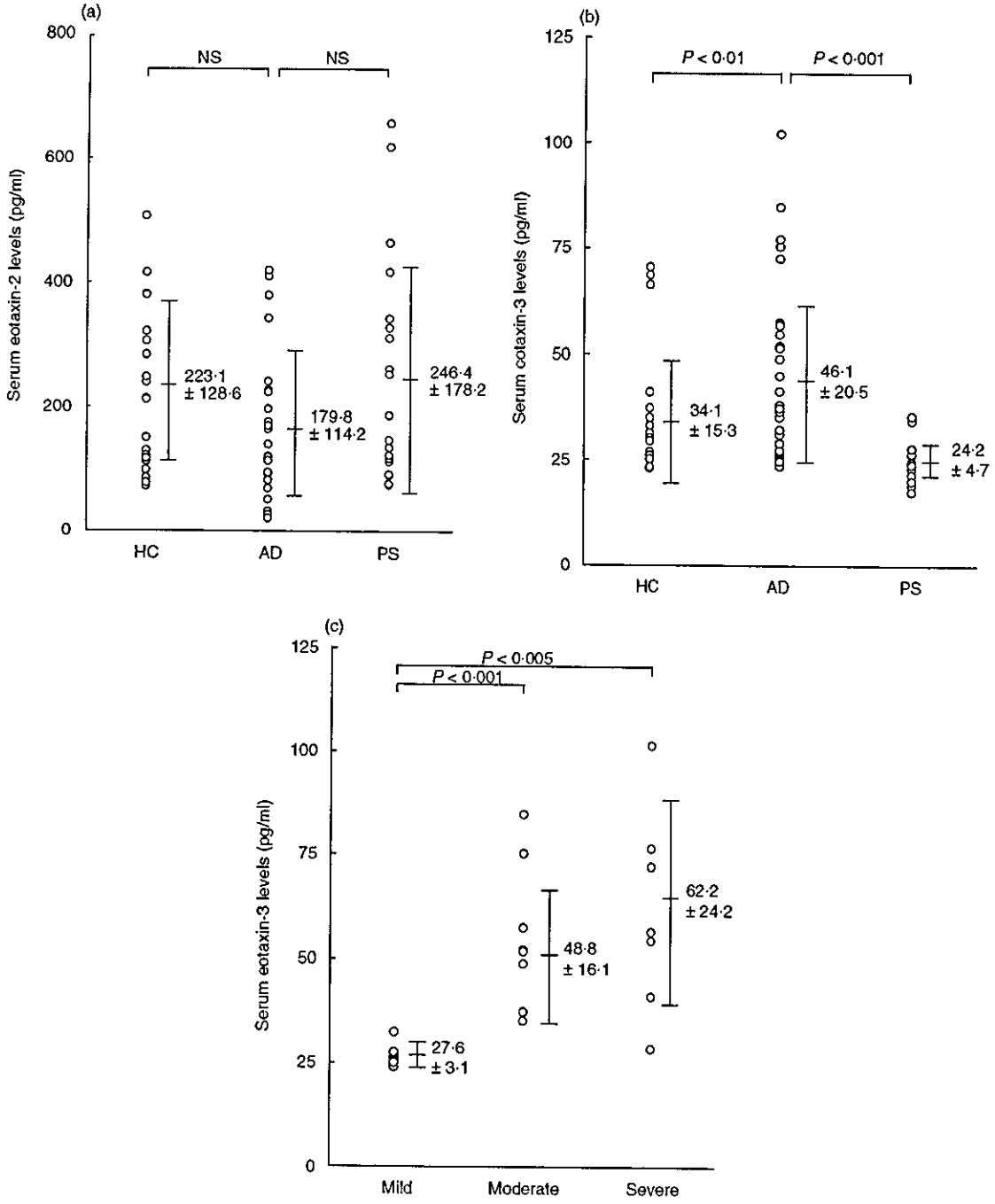

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) results of eotaxin-2/CCL24 (a) and eotaxin-3/CCL26 (b) using sera of patients with atopic dermatitis (AD), patients with psoriasis vulgaris and control subjects. (c) Serum eotaxin-3/CCL26 levels of the three groups (mild, moderate and severe) of patients with AD.

levels, the serum MDC/CCL22 levels and eosinophil numbers in peripheral blood. This indicates that the serum levels of eotaxin-3/CCL26 are also correlated with the disease activity of AD. Because we found a wide range in the eotaxin-3/CCL26 levels of patients with AD, we analysed the association between serum eotaxin-3/CCL26 levels and disease duration of AD. However, we did not find any significant association (data not shown). This is the first report describing the serum eotaxin-3/CCL26 and its possible role in the disease activity of AD. We also found that serum eotaxin-3/CCL26 levels were not significantly correlated with

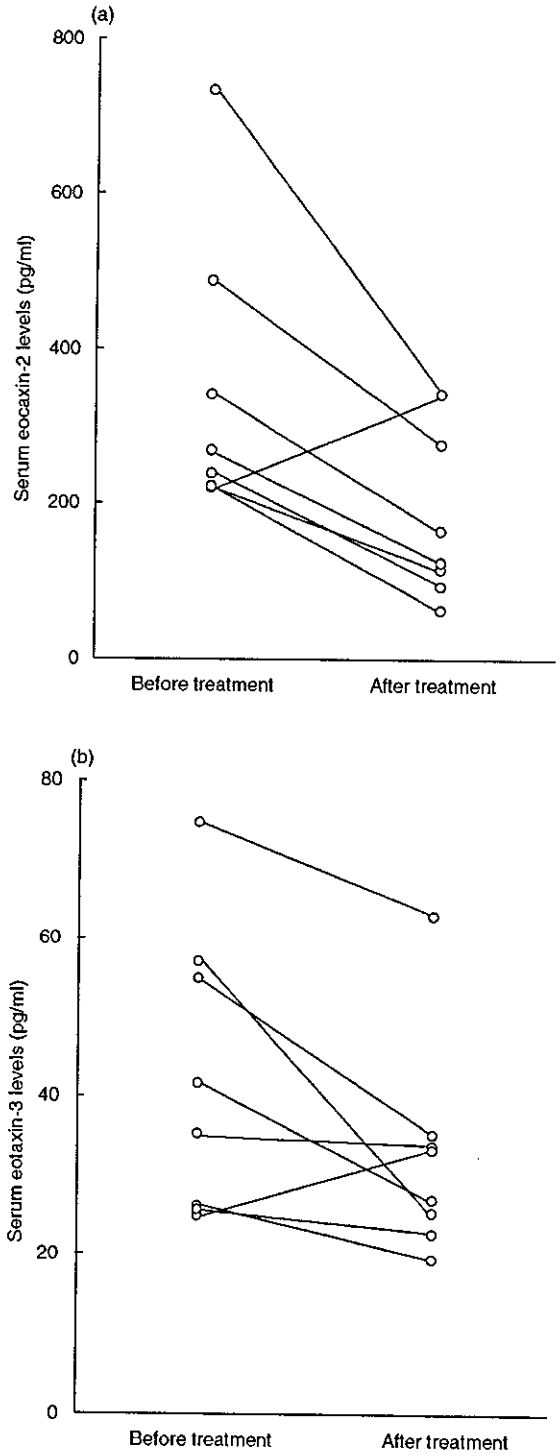

Fig. 2. Serum eotaxin-2/CCL24 (a) and eotaxin-3/CCL26 (b) levels of eight patients with atopic dermatitis (AD) were measured before and after treatment with topical corticosteroids and oral antihistamines.

Table 1. Correlation coefficient (r) among serum eotaxin-3/CCL26 levels and other clinical and laboratory data in patients with atopic dermatitis (AD)

	Eotaxin-3	
	r	P-value
TARC	0·50	< 0·05
MDC	0·46	< 0·05
Eosinophils	0·44	< 0·05
SCORAD	0·55	< 0·01
Eotaxin-2	0·21	0·25

MDC, macrophage-derived chemokine; SCORAD, scoring atopic dermatitis index; TARC, thymus and activation-regulated chemokine.

serum eotaxin-2/CCL24 levels, although both chemokines have similar biological functions in terms of recruitment of eosinophils via the CCR3. An *in vitro* study reported that eotaxin-3/CCL26 is strongly expressed and produced in vascular endothelial cells by stimulation with interleukin-4 (IL-4) [10], whereas eotaxin-2/CCL24 is not produced in vascular endothelial cells. Thus, the production of eotaxin-3/CCL26 is distinct from that of eotaxin-2/CCL24, which might explain the results of our *in vivo* data.

In conclusion, we have clearly shown that serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, are significantly correlated with the disease activity of AD. This strongly suggests that eotaxin-3/CCL26 might have an important role in the pathogenesis of AD in conjunction with TARC/CCL17 and MDC/CCL22.

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Increased serum cutaneous T cell–attracting chemokine (CCL27) levels in patients with atopic dermatitis and psoriasis vulgaris

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Background: Both atopic dermatitis (AD) and psoriasis vulgaris (PsV) are characterized as chronic and relapsing inflammatory skin diseases associated with various immunologic abnormalities. Cutaneous T cell–attracting chemokine (CTACK; CCL27) is a member of the CC chemokine family and a functional ligand for CC chemokine receptor 10. It is selectively expressed in skin and attracts CC chemokine receptor 10–expressing skin-homing memory T cells. The epidermal keratinocyte is a main source of CTACK, suggesting the involvement of various inflammatory skin diseases.

Objective: The purpose of this investigation was to clarify whether CTACK produced by keratinocytes is detected in the sera of patients with AD and PsV and to examine the correlation between the serum CTACK levels and disease activity of patients with AD and PsV.

Methods: We measured the serum CTACK levels in 50 patients with AD, 30 patients with PsV, and 22 healthy control subjects. We also divided 50 patients with AD into 3 groups (ie, those with mild, moderate, and severe disease) and compared them among 3 categories. Moreover, we compared the serum CTACK levels of patients with AD and PsV with clinical or laboratory data. Immunohistochemical staining of CTACK and IFN-induced protein of 10 kd (IP-10; CXCL10) was performed on the lesional skin of patients with AD and PsV.

Results: The serum CTACK levels in patients with AD and PsV were significantly higher than those in healthy control subjects. The serum CTACK levels in patients with AD significantly correlated with scoring atopic dermatitis (SCORAD) scores, serum soluble IL-2 receptor levels, serum soluble E-selectin levels, serum thymus and activation-regulated chemokine levels, and serum macrophage-derived chemokine levels. Serum CTACK levels in patients with PsV significantly correlated with the serum IP-10 levels but not with the Psoriasis Area and Severity Index score. Immunohistochemical staining showed CTACK was strongly expressed in lesional keratinocytes of patients with AD and PsV, whereas IP-10 was strongly expressed in lesional keratinocytes of patients with PsV and focally in those with AD.

Conclusion: These results suggest that CTACK might be one of the important chemokines for the pathogenesis of AD and PsV. (J Allergy Clin Immunol 2003;111:592-7.)

Key words: Cutaneous T cell–attracting chemokine, CCL27, atopic dermatitis, psoriasis vulgaris, disease activity

Atopic dermatitis (AD) is an inflammatory skin disease that is characterized by pruritic and eczematous lesions persisting chronically. It is often associated with increased serum IgE levels and peripheral blood eosinophilia. Histopathologically, the skin lesions in AD show infiltration of T lymphocytes, especially cutaneous lymphocyte antigen–positive memory T cells, as well as eosinophils and macrophages.[1,2] Previous studies revealed that serum soluble IL-2 receptor (sIL-2R) levels,[3] serum soluble E-selection levels,[4] serum thymus and activation-regulated chemokine (TARC) levels,[5] and serum macrophage-derived chemokine (MDC) levels[6] significantly correlate with the disease activity of AD, suggesting involvement in the pathogenesis of AD.

Psoriasis vulgaris (PsV) is also a chronic and relapsing inflammatory skin disease characterized as a T cell–mediated autoimmune disorder.[7] It has been proposed that interaction between T cells and epidermal keratinocytes plays a central role in the pathogenesis of PsV.[8]

Cutaneous T cell–attracting chemokine (CTACK; CCL27) belongs to the CC chemokine family and is a ligand for CC chemokine receptor 10.[9-11] It selectively chemoattracts cutaneous lymphocyte antigen–positive, CC chemokine receptor 10–positive memory T cells into the inflammatory sites.[9,12] It is reported that CTACK is expressed only in the skin but not in other organs and is especially expressed constitutively in epidermal keratinocytes.[9] In addition, previous in vitro data revealed that the expression and production of CTACK in keratinocytes is upregulated by stimulation with both IL-1β and TNF-α.[9,13,14] Although the quantitative PCR analysis of CTACK showed no significant differences between the lesional skin of patients with AD and PsV and normal skin,[13] on the basis of these results, CTACK might be

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Abbreviations used
AD: Atopic dermatitis
CTACK: Cutaneous T cell–attracting chemokine
CCR10: CC chemokine receptor 10
IP-10: IFN-induced protein of 10 kd
MDC: Macrophage-derived chemokine
PASI: Psoriasis Area and Severity Index
PsV: Psoriasis vulgaris
SCORAD: Scoring atopic dermatitis
sIL-2R: Soluble IL-2 receptor
TARC: Thymus and activation-regulated chemokine

associated with various inflammatory skin diseases, such as AD and PsV. Indeed, very recently, Homey et al[14] reported that CTACK is strongly expressed in the lesional keratinocytes of patients with AD compared with in the normal keratinocytes of healthy control subjects. However, it has not yet been clarified whether CTACK secreted from keratinocytes is found in the sera of patients with inflammatory skin diseases, such as AD or PsV.

In this study we measured the serum CTACK levels in patients with AD and PsV and compared them with those in healthy control subjects. We also compared the serum CTACK levels in patients with AD before and after treatment. Moreover, we examined the correlation between the serum CTACK levels in patients with AD and other laboratory data for disease activity of AD, such as serum sIL-2R, soluble E-selectin, serum TARC, and serum MDC levels and eosinophil numbers in peripheral blood. In patients with PsV, we also examined the correlation among the serum CTACK level, the Psoriasis Area and Severity Index (PASI) score, and the serum IFN-induced protein of 10 kd (IP-10; CXCL10) level. Furthermore, we performed immunohistochemical staining of CTACK in the lesional skin of patients with AD and PsV and compared the results with IP-10 expression in the lesional skin of patients with these diseases.

METHODS

Samples and reagents

The sera of 50 patients with AD (mean ± SEM age, 28.5 ± 6.8 years), 22 healthy control subjects (mean ± SEM age, 36.4 ± 8.5 years), and 30 patients with PsV (mean ± SEM age, 51.2 ± 8.0 years) were used in this study. All patients with AD were given a diagnosis according to the criteria of Hanifin and Rajka[1] and were treated with topical corticosteroids in combination with oral antihistamines. Twenty-six of 50 patients with AD had personal histories of respiratory allergy. Disease activity of AD was determined by using the modified scoring atopic dermatitis (SCORAD) index system.[15,16] In this study we divided the patients with AD into 3 groups (ie, those with mild, moderate, and severe disease) according to a proposal for severity grading of AD by using only objective criteria (mild disease, SCORAD score = <15; moderate disease, SCORAD score = 15-40; and severe disease, SCORAD score = >40).[16] Twenty-two healthy control subjects and 30 patients with PsV had no histories of allergy, and their total serum IgE levels were within the normal range. In addition, we estimated the clinical severity of symptoms in patients with PsV by using the PASI score. After venous blood was drawn, serum was separated and stored at −20°C until use.

For immunohistochemical staining, mouse anti-human CTACK mAb (R&D Systems), mouse IgG (R&D Systems), rabbit anti-human IP-10 antibody (Santa Cruz Biotechnology Inc), and rabbit IgG (Jackson ImmunoResearch) were used.

ELISA protocol for CTACK

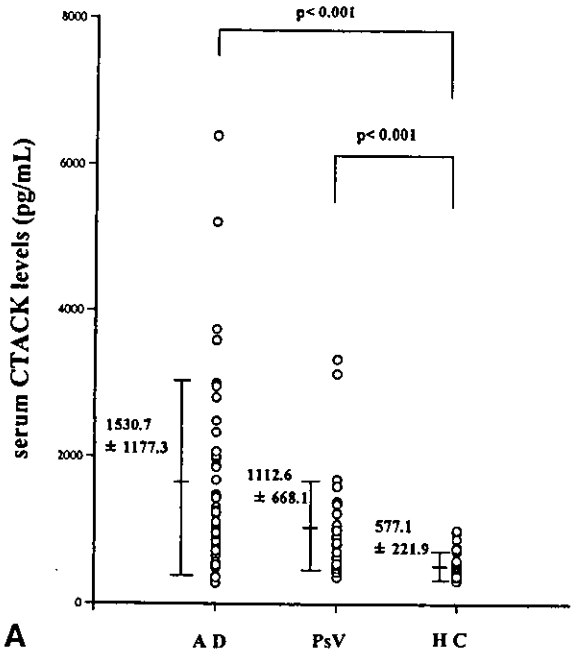
In this study recombinant human CTACK (R&D Systems), anti-human CTACK mAb (clone 124308, R&D Systems) and biotinylated anti-human CTACK polyclonal antibody (R&D Systems) were used. Two nanograms of anti-human CTACK mAb was applied to each well of a 96-well microplate (Costar Co) and coated overnight at room temperature. Wells were then washed 3 times and blocked for at least 1 hour with 300 μL of PBS containing 1% BSA (Intergen Co), 5% sucrose, and 0.05% NaN_3. Subsequently, standard dilutions of recombinant CTACK (2000, 1000, 500, 250, 125, 62.5, 31.2, and 0 pg/mL) and samples were added and incubated for 2 hours at room temperature. After washing as above, each well was incubated for 2 hours with 100 μL of biotinylated anti-human CTACK antibody diluted 200-fold in TBS containing 0.1% PBS, 0.05% Tween-20, and 2% normal goat serum (Sigma chemical Co). After washing as above and staining with streptavidin–horseradish peroxidase (R&D Systems) diluted 200-fold in PBS containing 1% BSA for 20 minutes, 100 μL of substrate solutions A and B (R&D Systems) mixed 1:1 was added to each well. Fifteen minutes after substrate addition, the reaction was stopped by the addition of 50 μL of 2N H_2SO_4, and the OD_{450} was measured with a microplate reader (Model 550, Bio-Rad). Recombinant human CTACK was used for standard curves. Data of CTACK levels obtained from the ELISA are presented as means ± SD.

ELISA for sIL-2R, soluble E-selectin, TARC, MDC, and IP-10

The serum levels of sIL-2R, soluble E-selectin, TARC, MDC, and IP-10 were measured by using a commercially available ELISA system. A 96-well microplate coated with either mAb against human sIL-2R (R&D Systems), soluble E-selectin (MedSystems), TARC (Techne Corp), MDC (R&D Systems), or IP-10 (R&D Systems) was used. Each sample was added and incubated. After the removal of unbound material by means of aspiration and washing, a conjugate with sIL-2R, soluble E-selectin, TARC, MDC, or IP-10 was detected on the basis of its reaction with a substrate solution, which yielded a colored product proportional to the amount of conjugate. The OD_{450} was measured with a microplate reader. Data obtained from the ELISA are presented as means ± SD.

Immunohistochemical staining of CTACK and IP-10 in the lesional skin of patients with AD and PsV and the normal skin of healthy control subjects

Biopsy samples were taken from the lesional skin of patients with AD (n = 3) and PsV (n = 3) and the normal skin of healthy control subjects (n = 2). The obtained skin samples were embedded in Tissue-Tek OCT compound (Miles Inc) for at least 24 hours at −80°C. These frozen sections were cut with a cryostat set at 6 μm of thickness and fixed in 4% paraformaldehyde. They were incubated overnight with mouse anti-human CTACK mAb, mouse IgG, rabbit anti-human IP-10 antibody, or rabbit IgG at 4°C after the endogeneous peroxidase activity. The samples were then incubated with biotin-conjugated anti-mouse IgG (DAKO Co) or biotin-conjugated anti-rabbit IgG (DAKO Co) for 30 minutes. Subsequently, the samples were washed with PBS, incubated with ABC complex followed by diaminobendizine solution until brown staining was visible, and counterstained with Mayer hematoxylin, according to the manufacturer's instructions.



Statistical analysis

The data were analyzed by using the Mann-Whitney *U* test. Correlation coefficients were determined by using the Spearman rank correlation test. *P* values of less than .05 were considered to be statistically significant.

RESULTS

Serum CTACK levels in patients with AD and PsV

The mean ± SD serum CTACK levels in patients with AD and PsV were 1530.7 ± 1177.3 and 1112.6 ± 668.1 pg/mL, respectively, whereas the level in healthy control subjects was 577.1 ± 221.9 pg/mL. The serum CTACK levels in patients with AD and PsV were significantly higher than those in healthy control subjects ($P < .001$, respectively; Fig 1, *A*). In addition, we measured the serum CTACK levels in 10 patients with AD before and after treatment with a topical corticosteroid in combination with oral antihistaminic drugs. Before treatment, the serum CTACK levels were 2680.4 ± 851.3 pg/mL, whereas they decreased to 860.5 ± 352.1 pg/mL after treatment. There was a significant difference between levels before and after treatment ($P < .01$; Fig 1, *B*).

Serum IP-10 levels in patients with AD and PsV

The mean ± SD serum IP-10 levels in patients with AD and PsV were 124.55 ± 41.95 and 199.50 ± 129.78 pg/mL, respectively. In addition, the level in healthy control subjects was 87.70 ± 27.76 pg/mL. The serum IP-10 levels in patients with PsV were significantly higher than those in healthy control subjects ($P < .05$), whereas the serum IP-10 levels in patients with AD were not significantly higher than those of healthy control subjects. Moreover, there was no significant difference in the serum IP-10 levels between patients with AD and those with PsV.

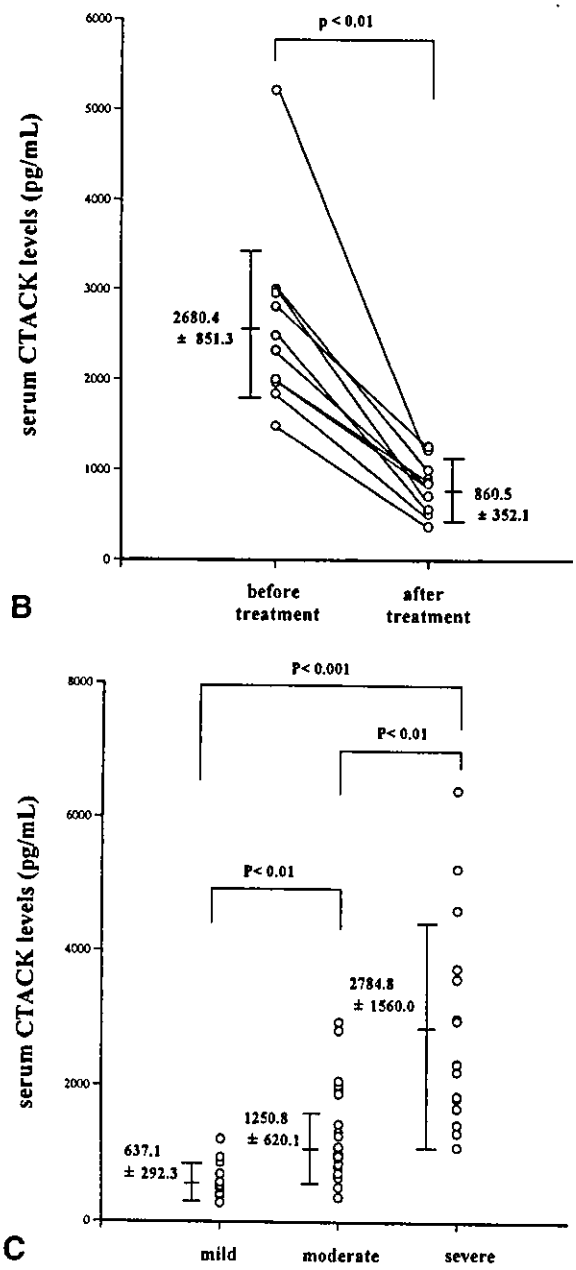


FIG 1. A, Serum CTACK levels in patients with AD (n = 50), patients with PsV (n = 30), and healthy control subjects (*HC*; n = 22). B, Serum CTACK levels in patients with AD (n = 10) were evaluated before and after treatment. C, Comparison of serum CTACK levels among healthy control subjects and 3 groups of patients with AD: those with mild (n = 10), moderate (n = 24), and severe disease (n = 16).

Serum CTACK and IP-10 levels in patients with AD and PsV and disease activity

The mean ± SD CTACK levels in the mild, moderate, and severe groups of patients with AD were 637.1 ± 292.3, 1250.8 ± 620.1, and 2784.8 ± 1560.0 pg/mL, respectively. The serum CTACK levels in the severe group were significantly higher than those in the mild group ($P < .001$) or the moderate group ($P < .01$), indi-

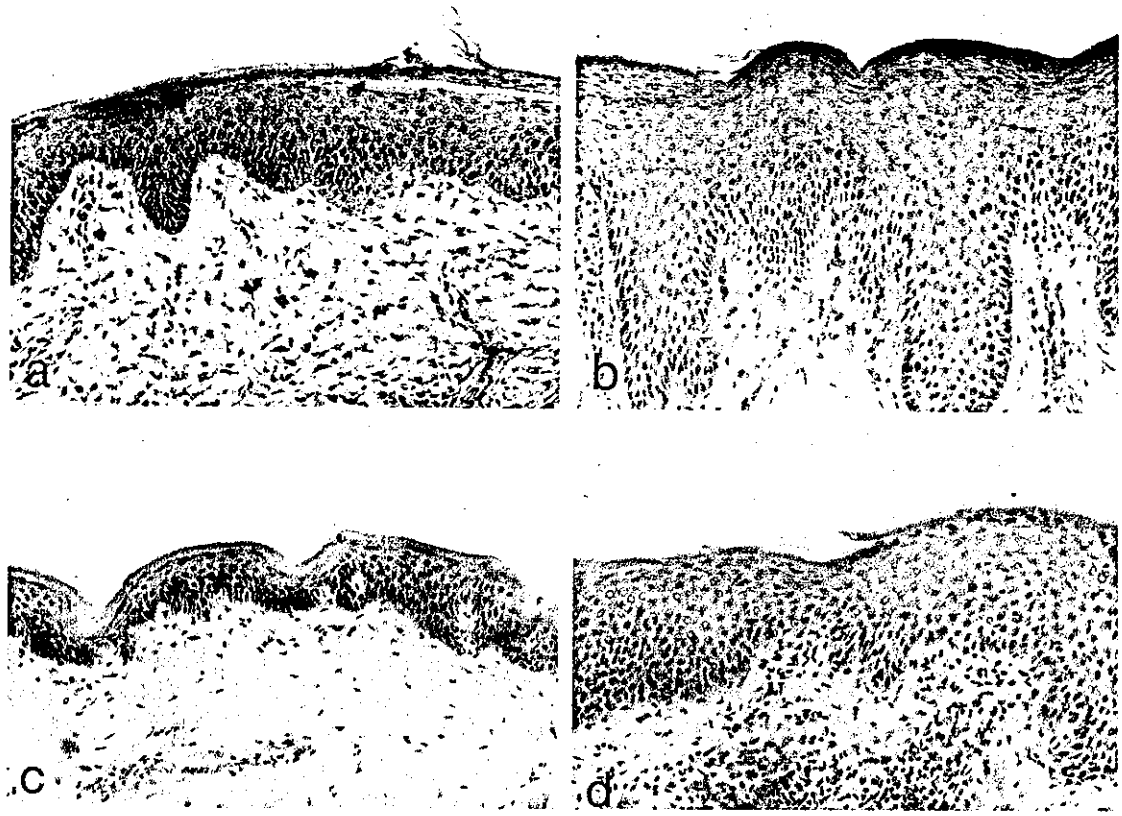


FIG 2. CTACK was strongly expressed in the lesional keratinocytes of patients with AD (**a**) and PsV (**b**). In contrast, it was moderately expressed in the basal layer of the normal skin of healthy control subjects (**c**). When an isotype-matched control was used, no reactivity was observed (**d**; original magnification: 200×).

cating that they increased in accordance with the disease severity (Fig 1, *C*). Moreover, we compared the serum CTACK levels with the clinical and laboratory data that were previously reported to indicate the disease activity of AD. The serum CTACK levels significantly correlated with the SCORAD score ($r = 0.70$, $P < .001$), serum sIL-2R levels ($r = 0.74$, $P < .001$), serum MDC levels ($r = 0.85$, $P < .001$), serum TARC levels ($r = 0.58$, $P < .001$), serum soluble E-selectin levels ($r = 0.56$, $P < .002$), and eosinophil numbers in peripheral blood ($r = 0.62$, $P < .001$). However, there was no significant correlation between the serum CTACK levels and the serum IP-10 levels because the average levels of serum IP-10 in patients with AD were the same as those in healthy control subjects. We also compared the serum CTACK levels in patients with PsV with the serum IP-10 levels and the PASI score. The serum CTACK levels significantly correlated with the serum IP-10 levels ($r = 0.54$, $P < .002$) but not with the PASI score. The results between the serum CTACK levels and other data are summarized in Table I.

Immunoreactive CTACK and IP-10 in the lesional skin of patients with AD and PsV

In the lesional skin of patients with AD and PsV, immunohistochemical staining revealed that CTACK was strongly expressed almost completely in the keratinocytes (Fig 2, *a* and *b*). By contrast, in the normal skin of healthy control subjects, CTACK was moderately expressed in the basal cells but not in the squamous cells (Fig 2, *c*). When an isotype-matched control was used, no positive staining was observed (Fig 2, *d*). IP-10 was strongly detected in all layers of lesional keratinocytes of patients with PsV and focally in the lesional keratinocytes of patients with AD (Fig 3). No reactivity was observed when the isotype-matched control was used (data not shown).

DISCUSSION

CTACK is produced in keratinocytes,[9-11,13] especially after stimulation with TNF-α and IL-1β in vitro. In this study we investigated whether CTACK is detected in the sera of patients with AD and PsV and found that the serum CTACK levels in patients with AD and PsV were significantly greater than those in healthy control subjects. This result indicates that CTACK secreted by lesional keratinocytes can be detectable in the sera of patients with these inflammatory skin diseases. We also showed that in patients with AD, the serum CTACK levels increased in accordance with the severity of AD. Previous studies reported that the serum sIL-2R,[3] E-selectin,[4] TARC,[5] and MDC[6] levels correlated with the disease activity of AD. Our study further clarified that the serum CTACK levels significantly correlated with these

TABLE I. Correlation coefficients between the serum CTACK levels and other clinical and laboratory data in patients with AD and PsV

	CTACK	
	r	*P* value
AD		
MDC	0.85	<.001
sIL-2R	0.74	<.001
SCORAD	0.70	<.001
Eosinophils	0.61	<.001
TARC	0.58	<.001
Soluble E-selectin	0.56	<.002
PsV		
IP-10	0.58	<.009
PASI	0.07	.17

Eosinophils, Numbers of eosinophils in peripheral blood.

markers, as well as with the SCORAD score, suggesting that the serum CTACK levels also correlate with the disease activity of AD. This is the first report describing a correlation between serum CTACK levels and the disease activity of AD, and we suggest that it might be useful to measure serum CTACK levels when estimating the severity of other inflammatory skin diseases. Immunohistochemical staining of CTACK in the lesional skin of patients with AD and PsV was consistent with the findings of a previous report that lesional keratinocytes abundantly produce CTACK in patients with inflammatory skin disease.[14] Thus the high serum levels and the strong expression of CTACK in lesional keratinocytes suggest that CTACK might play an important role in the pathogenesis of AD and PsV.

Previously, Giustizieri et al[17] showed that IP-10 was highly detected in the lesional keratinocytes of patients with PsV, whereas it was weakly detected in the lesional keratinocytes of those with AD. In this study we confirmed that IP-10 was strongly expressed in the lesional keratinocytes of patients with PsV, whereas it was focally expressed in those with AD. Moreover, the serum IP-10 levels in patients with PsV, but not those with AD, were significantly higher than those in healthy control subjects and correlated with the serum CTACK levels. However, there was no significant difference in serum IP-10 levels between patients with AD and those with PsV. Accumulated evidence revealed that IP-10 was secreted from not only keratinocytes but also from various other cell types[18] and was also detected in the supernatants of PBMCs in vitro.[19] Thus we assume that the serum IP-10 levels reflect the total amount of secretion from not only skin cells but also peripheral blood cells. This is why data on the serum IP-10 levels did not correspond to those of immunohistochemistry of the skin in contrast to CTACK. In addition, both the serum IP-10 levels and the serum CTACK levels did not correlate with the PASI score, which is considered to be available for quantifying the extent of the clinical symptoms of PsV.[20] Because the number of patients with PsV was smaller than the number of patients with AD, increased numbers will be required to make a conclusion concerning the correlation between serum CTACK levels, serum IP-10 levels, and disease severity.

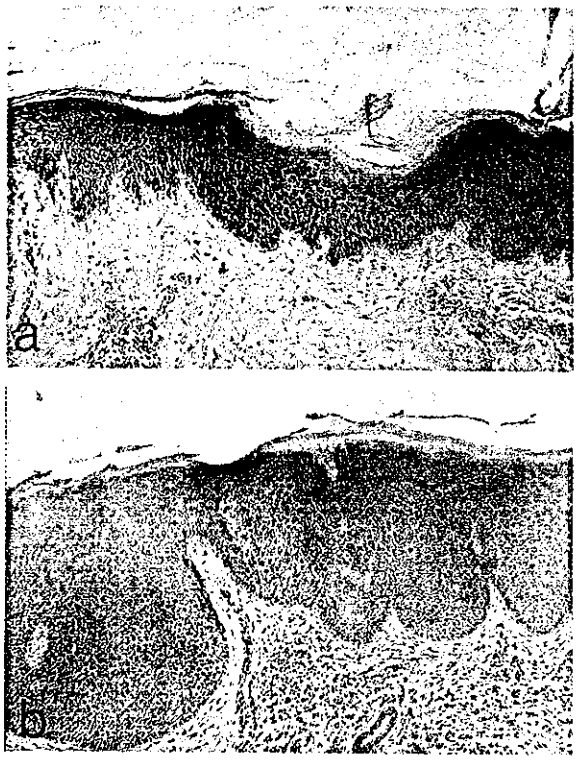


FIG 3. IP-10 was focally expressed in the lesional keratinocytes of patients with AD (**a**), whereas it was strongly expressed in those with PsV (**b**). When an isotype-matched control was used, no reactivity was observed (data not shown; original magnification: 100×).

In conclusion, serum CTACK levels were significantly higher in patients with AD and PsV than in healthy control subjects and significantly correlated with the disease activity of AD but not PsV. In addition, immunoreactive CTACK was strongly detected in the lesional keratinocytes of patients with AD and PsV. These results strongly suggest that CTACK might be one of the important chemokines in the lesional formation of AD and PsV but might have a distinct role in AD and PsV.

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Cutaneous Biology

High level of thymus and activation-regulated chemokine in blister fluid and sera of patients with bullous pemphigoid

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Summary

Background Bullous pemphigoid (BP) is an autoimmune blistering disease characterized by eosinophilia and high serum IgE levels. The accumulated evidence suggests that various cytokines are involved in the lesional skin of patients with BP. Recently, thymus and activation-regulated chemokine (TARC/CCL17), a CC chemokine, was identified as a selective chemoattractant for CC chemokine receptor 4 (CCR4)-expressing cells.

Objective In this study, we examined the involvement of TARC in patients with BP.

Methods We determined the fluid and serum TARC levels in patients with BP by enzyme-linked immunosorbent assay and compared the serum TARC levels with the eosinophil numbers in peripheral blood. We also compared the serum TARC levels in five patients with BP before and after they were treated. Moreover, we examined TARC, CCR4 and CXC chemokine receptor 3 (CXCR3) expression in the lesional skin of patients with BP by immunohistochemical procedures. Furthermore, we measured CCR4 positivity in CD4+ CD45RO+ cells of peripheral blood mononuclear cells (PBMCs) in patients with BP and healthy control subjects.

Results The fluid TARC levels in patients with BP were significantly higher than those in blisters from burn patients or suction blisters of healthy control subjects. The serum TARC levels in patients with BP were also significantly higher than those in pemphigus vulgaris (PV) patients and healthy control subjects, and decreased after the treatment. The serum TARC levels in patients with BP significantly correlated with the eosinophil numbers in peripheral blood ($r = 0{\cdot}72$, $P < 0{\cdot}002$). Immunohistochemistry showed a strong reactivity of TARC in the epidermal keratinocytes (KCs) of BP. Moreover, both CCR4 and CXCR3 were expressed on the dermal infiltrating CD4+ T cells mainly beneath the bullae of patients with BP. Fluorescence-activated cell sorting analysis showed a higher percentage of CCR4 positivity in CD4+ CD45RO+ cells of PBMCs in patients with BP than that in healthy control subjects, while there was no significant difference of CXCR3 positivity in CD4+ CD45RO+ cells of PBMCs between patients with BP and healthy control subjects.

Conclusions These findings strongly suggest that TARC may be one of the important chemokines that are involved in the pathogenesis of BP.

Key words: bullous pemphigoid, CCR4, keratinocytes, thymus and activation-regulated chemokine

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering skin disease, usually occurring in the elderly, which is characterized by large, tense blisters and the immunopathological finding of C3 and IgG at the epidermal basement membrane zone with circulating antibasement membrane zone antibodies. Most patients with circulating antibasement membrane IgG have antibasement membrane IgE in their sera.[1] In addition to the immunopathological features, almost

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one-half of patients had elevated total IgE, which positively correlated with IgG antibasement membrane zone immunofluorescence titre.[2] Elevated levels of IgE also correlated with the presence of pruritus. Moreover, about one-half of patients have peripheral blood eosinophilia, sometimes marked.

Previous studies have revealed that all patients with BP have autoantibodies against the 230 kDa molecule (BPAG1), which has been localized ultrastructurally to the plaque of the hemidesmosome exactly where keratin intermediate filaments insert.[3] Moreover, most patients with BP have autoantibodies against 180 kDa molecule (BPAG2).[4] BPAG2 is located just at the cell membrane, partially inside and partially outside, typical of a transmembrane molecule.[4] The pathogenic relevance of anti-BP180 antibodies has been shown using a passive transfer model of BP, in which neonatal mice are injected with antibodies that recognize a segment of murine BPAG2 protein that is homologous with the human NC16A domain.[5] Further studies of epitope mapping of BPAG2 revealed that the NC16A domain of BPAG2 is the major site recognized by BP sera.[6] Recently, Budinger *et al.*[7] revealed the existence of autoreactive T cells that recognize the extracellular domain of BPAG2 in peripheral blood of patients with BP. They reported that these autoreactive T cells participate in blister formation. These autoreactive T-cell lines and clones show the characteristics of T helper (Th)2 rather than Th1.[7] Consistent with these data, a recent study of the subclass distribution of antibodies to full-length BPAG2 and recombinant fragments of this domain reported that IgG4 and IgE are the major immunoglobulins that preferentially react with two distinct epitopes within BPAG2.[8] On the other hand, Lin *et al.*[9] have reported that autoreactive T cells from patients with BP recognize the BPAG2 NC16A domain and that they show the features of Th2 as well as Th1. With regard to cytokine profiles from blister fluid and sera of patients with BP, varying results are reported.[10–14] For example, Kaneko *et al.*[11] found high levels of interferon (IFN)-γ in blister fluid, whereas Ameglio *et al.*[12] reported high levels of interleukin (IL)-4, IL-5 and IL-6 in blister fluid. Thus, the features of BP, in terms of Th1/Th2-type cytokine profiles, need further clarification.

It has been reported that chemokine receptor expression varies considerably in lymphocytes. Th1- and Th2-type cells differ in their chemokine receptor expression and their responsiveness to chemokines, which may explain the phenomenon of Th1- or Th2-forming cell infiltration in inflammatory sites. Th2-type cells selectively express CC chemokine receptor 3 (CCR3), CCR4 and CCR8,[15,16] while Th1-type cells selectively express CXC chemokine receptor 3 (CXCR3).[17] CC chemokines such as thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22), which are functional ligands for CCR4,[18,19] have been reported to be expressed in various allergic diseases such as atopic dermatitis (AD)[20,21] and bronchial asthma.[22] In previous papers, we have shown: (i) that TARC and MDC are highly expressed in NC/Nga mouse lesional skin, which is regarded as a mouse model of human AD;[23] (ii) that CCR4 is preferentially expressed on CD4+ cells of peripheral blood from AD patients and reflects disease activity;[24] (iii) that TARC is strongly expressed in epidermal keratinocytes (KCs) of lesional skin of AD patients; and (iv) that serum TARC level also reflects the disease activity of AD patients.[21] As AD is known to be a Th2-dominant disease, characterized by elevated serum IgE levels and peripheral blood eosinophilia, we decided to investigate the TARC levels in sera and blister fluid of patients with BP by enzyme-linked immunosorbent assay (ELISA). We also examined the expression of TARC, CCR4 and CXCR3 in lesional skin of BP by immunohistochemical procedures. Finally, we measured CCR4 and CXCR3 positivity in CD4+ CD45RO+ cells of peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS) analysis in patients with BP.

Materials and methods

Subjects

Twenty patients with BP who came to our hospitals within a 10-year period were enrolled in this study. BP was diagnosed on the basis of clinical features, findings of skin biopsy examinations by light microscopy, direct immunofluorescence and indirect immunofluorescence test on salt-split human skin (IgG deposition at the epidermal roof of the split). Eight samples of BP blister fluid were collected from eight BP subjects (cases 1–8; three men and five women, 19–93 years of age) before treatment. As controls, fresh burn blister fluid from three patients (women, 19–48 years of age) and suction blister fluid from three healthy control subjects (two men and one woman, 34–40 years of age) were also collected.

Serum samples were collected from 13 patients with BP (seven men and six women, 19–86 years of age) and peripheral blood eosinophil numbers were coun-

ted. As controls, serum samples were collected from eight patients with pemphigus vulgaris (PV) (two men and six women, 47–88 years of age) and from eight healthy control subjects (seven men and one woman, 26–41 years of age). PV was diagnosed on the basis of clinical features, findings of skin biopsy examination by light microscopy and direct and indirect immunofluorescence tests. The samples collected were stored at −80 °C until use.

Measurement of fluid and serum thymus and activation-regulated chemokine levels by enzyme-linked immunosorbent assay

Immunoreactive TARC in blister fluids and sera was quantified by a human TARC ELISA kit (Genzyme Corp., Cambridge, MA, U.S.A.). These assays employ the quantitative sandwich enzyme immunoassay technique. Optical densities were measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). The concentrations of the chemokines were calculated from the standard curve generated by a curve-fitting program. The minimum detectable dose of TARC was 7 pg mL^{-1}. Data of TARC levels obtained from the ELISA are presented as mean ± SD.

Samples and reagents for immunostaining

Purified antihuman CCR4 monoclonal antibody (mAb) (mouse IgG1) was used for immunostaining. This mAb was characterized in terms of its antigen specificity[25] and was used for the immunohistochemical staining. Purified antihuman CXCR3 mAb (mouse IgG1; PharMingen, San Diego, CA, U.S.A.) and biotinylated antihuman CD4 mAb (mouse IgG1; Ancell Co., Bayport, MN, U.S.A.) were also used. Mouse IgG1 and biotinylated-mouse IgG1 (PharMingen) were used as negative controls. Purified antihuman TARC antibody (rabbit IgG; Peprotec, Rocky Hill, NJ, U.S.A.) and rabbit IgG (Jackson ImmunoResearch Lab. Inc., West Grove, PA, U.S.A.) were also used. To examine CCR4 and CXCR3 positivity in CD4+ CD45RO+ cells of PBMCs, the following antibodies were also used; phycoerythrin (PE)-conjugated antihuman CD45RO mAb (mouse IgG2a; PharMingen), Cy-Chrome™-conjugated antihuman CD4 mAb (mouse IgG1; PharMingen), fluorescein isothiocyanate (FITC)-conjugated antihuman CXCR3 mAb (mouse IgG1; PharMingen) and FITC-conjugated antihuman CCR4 mAb (mouse IgG1). As negative controls, FITC-conjugated mouse IgG1 (PharMingen), Cy-Chrome-conjugated mouse IgG1 (PharMingen) and PE-conjugated mouse IgG2a (R&D systems, Minneapolis, MN, U.S.A.) were used.

Immunohistochemical staining of lesional skin of patients with bullous pemphigoid and normal skin of healthy control subjects

We performed immunohistochemical staining of TARC, CCR4 and CXCR3 in the lesional skin of patients with BP ($n = 4$) and of normal skin ($n = 2$). Briefly, biopsied tissues from patients with BP were cut into 5 μm-thick cryostat sections and fixed in acetone. These sections were then stained with rabbit antihuman TARC antibody, rabbit IgG, mouse antihuman CCR4 mAb, mouse antihuman CXCR3 mAb or mouse IgG followed by ABC staining (Vector Lab. Inc., Burlingame, CA, U.S.A.). Diaminobenzidine was used for visualizing the staining, and counterstaining with Mayer haematoxylin was performed, according to the manufacturer's instructions. In order to study the relationship between CCR4, CXCR3 and CD4, the sections were examined by serial sectioning. The first section was stained with CCR4 or CXCR3, and the next section was stained with CD4.

Measurement of CCR4 and CXCR3 positivity in CD4+ CD45RO+ cells of peripheral blood mononuclear cells in patients with bullous pemphigoid and healthy control subjects

PBMCs were isolated from peripheral blood samples of three patients with BP and three healthy control subjects. PBMCs were isolated by means of centrifugation on a Ficoll-Metrizoate density gradient (Pharmacia Biotech, Uppsala, Sweden) at 450 *g* for 20 min at room temperature. PBMCs were washed three times with phosphate-buffered saline (PBS) and divided into tubes containing 3×10^5 cells each. CCR4 or CXCR3 positivity in CD4+ CD45RO+ cells was determined as previously described.[25]

Briefly, isolated PBMCs were stained with FITC-conjugated CCR4 mAb, or FITC-conjugated CXCR3 mAb, Cy-Chrome-conjugated CD4 mAb and PE-conjugated CD45RO mAb. FITC-conjugated mouse IgG1, Cy-Chrome-conjugated mouse IgG1 or PE-conjugated mouse IgG2a was also used as a negative control. The samples were analysed on a FACSCalibur (Becton Dickinson, San Diego, CA, U.S.A.) by using propidium iodide to exclude dead cells.

Statistical analyses

Statistical analyses were performed using the Mann–Whitney *U*-test for comparison of TARC levels between BP blister fluid, burn blister or suction blister fluid of healthy control subjects. $P < 0.05$ was considered to be statistically significant. In addition, correlation coefficients (r) were determined by the Spearman rank correlation test. $P < 0.05$ was considered to indicate significant correlation.

Results

Thymus and activation-regulated chemokine levels in bullous pemphigoid blister fluid were higher than those in blister fluid from burn patients or suction blisters of healthy control subjects

BP blister fluid contained 4258·0 ± 1865·1 pg mL^{-1} (range, 481·7–6579·7) of TARC, while blister fluid from burn patients contained 43·3 ± 6·9 pg mL^{-1} (range, 35·6–48·7 pg mL^{-1}) and suction blister fluid of healthy control subjects contained 64·5 ± 28·7 pg mL^{-1} (range, 46·0–97·6 pg mL^{-1}) of TARC (Fig. 1a). The fluid TARC levels in patients with BP were significantly higher than those in blister fluid from burn patients or suction blister fluid of healthy control subjects ($P < 0.005$, respectively). No significant correlation, however, was observed between each TARC level and antibody titre obtained from indirect immunofluorescence tests in BP subjects (data not shown).

Serum thymus and activation-regulated chemokine levels in patients with BP were significantly higher than those in patients with pemphigus vulgaris or healthy control subjects, and significantly correlated with eosinophil numbers in peripheral blood

Immunoreactive serum TARC levels in patients with BP were 1151·5 ± 885·6 pg mL^{-1} (range, 91·1–3981·4). In contrast, the serum TARC levels in PV patients and healthy controls were 270·9 ± 112·5 pg mL^{-1} (range, 113·0–407·0) and 196·6 ± 129·7 pg mL^{-1} (range, 65·8–404·1), respectively (Fig. 1b). Thus, the serum TARC levels in patients with BP were significantly higher than those in PV patients or healthy control subjects ($P < 0.002$, respectively). We also compared serum TARC levels in five patients with BP before and after treatment with oral corticosteroid. Serum TARC levels decreased significantly during the treatment (2200·0 ± 1364·5 pg mL^{-1} vs. 524·2 ± 395·6 pg mL^{-1}) in accordance with the improvement of skin conditions ($P < 0.01$) (Fig. 1c).

In addition, we compared serum TARC levels in patients with BP with eosinophil numbers in peripheral blood. There was a significant correlation between serum TARC levels and eosinophil number in peripheral blood ($r = 0.72$, $P < 0.002$) (Fig. 1d).

No significant correlation was observed between serum TARC levels and antibody titres obtained from indirect immunofluorescence tests in BP subjects (data not shown).

Immunoreactive thymus and activation-regulated chemokine, CCR4 and CXCR3 were observed in the lesional skin of patients with bullous pemphigoid

In the lesional skin of patients with BP, strong immunoreactivity of TARC was observed in the epidermal KCs, especially in the basal layer (Fig. 2a). Non-bullous lesions in patients with BP expressed TARC in a similar fashion (Fig. 2b). In contrast, no immunoreactivity for TARC was detected in normal skin (Fig. 2c). When the isotype matched control of TARC was used, no positive staining was observed (Fig. 2d). CCR4+ cells (Fig. 3a) and CXCR3+ cells (Fig. 3b) were observed mainly beneath the bullae in lesional skin. These CCR4+ or CXCR3+ cells were positive for CD4 (data not shown). When isotype matched controls of CCR4 or CXCR3 were used, no positive staining was observed (data not shown).

CCR4 positivity in CD4+ CD45RO+ cells of peripheral blood mononuclear cells in patients with bullous pemphigoid was higher than that in healthy control subjects, whereas there was no significant difference of CXCR3 positivity in CD4+ CD45RO+ cells of peripheral blood mononuclear cells between patients with bullous pemphigoid and healthy control subjects

CCR4 positivity in CD4+ CD45RO+ cells of PBMCs in patients with BP was 15·0 ± 2·7%, while that in healthy control subjects was 5·2 ± 1·8%. CCR4 positivity in CD4+ CD45RO+ cells of PBMCs in patients with BP was significantly higher than that in healthy control subjects ($P < 0.03$). CXCR3 positivity in CD4+ CD45RO+ cells of PBMCs in patients with BP and healthy control subjects was 29·2 ± 2·2% and 24·5 ± 3·5%, respectively. There was no significant difference between them. These results are summarized in Table 1.

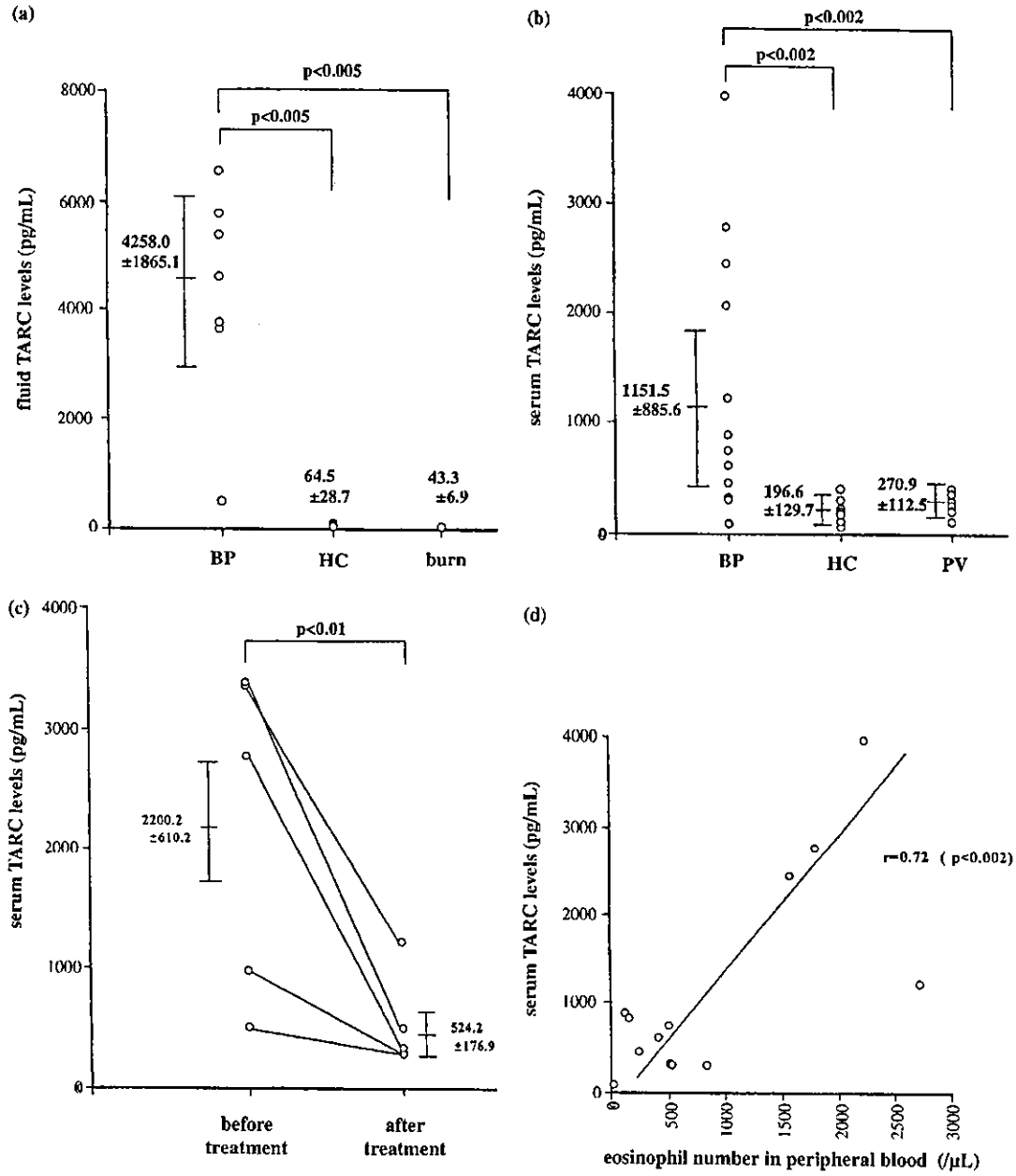

Figure 1. (a) Fluid thymus and activation-regulated chemokine (TARC) levels in patients with bullous pemphigoid (BP) (n = 8), burn patients (burn) (n = 3) and suction blisters of healthy control subjects (HC) (n = 3). Fluid TARC levels in patients with BP were significantly higher than those in burn patients or healthy control subjects (P < 0·005, P < 0·005, respectively). A dotted line indicates +2 SD of the fluid levels in healthy control subjects. (b) Serum TARC levels of patients with BP (n = 13), pemphigus vulgaris (PV) patients (n = 8) and healthy control subjects (n = 8). Serum TARC levels in patients with BP were significantly higher than those in PV patients or healthy control subjects (P < 0·002, P < 0·002, respectively). A dotted line indicates mean +2 SD of the serum levels in healthy control subjects. (c) Comparison of serum TARC levels in patients with BP (n = 5) between before and after treatment with oral and topical corticosteroid treatment. Serum TARC levels were significantly decreased after the treatment (P < 0·01). (d) Comparison between serum TARC levels in patients with BP and eosinophil numbers in peripheral blood (n = 13). A significant correlation was shown between these laboratory data (r = 0·72, P < 0·002). Each bar indicates mean ± SD.

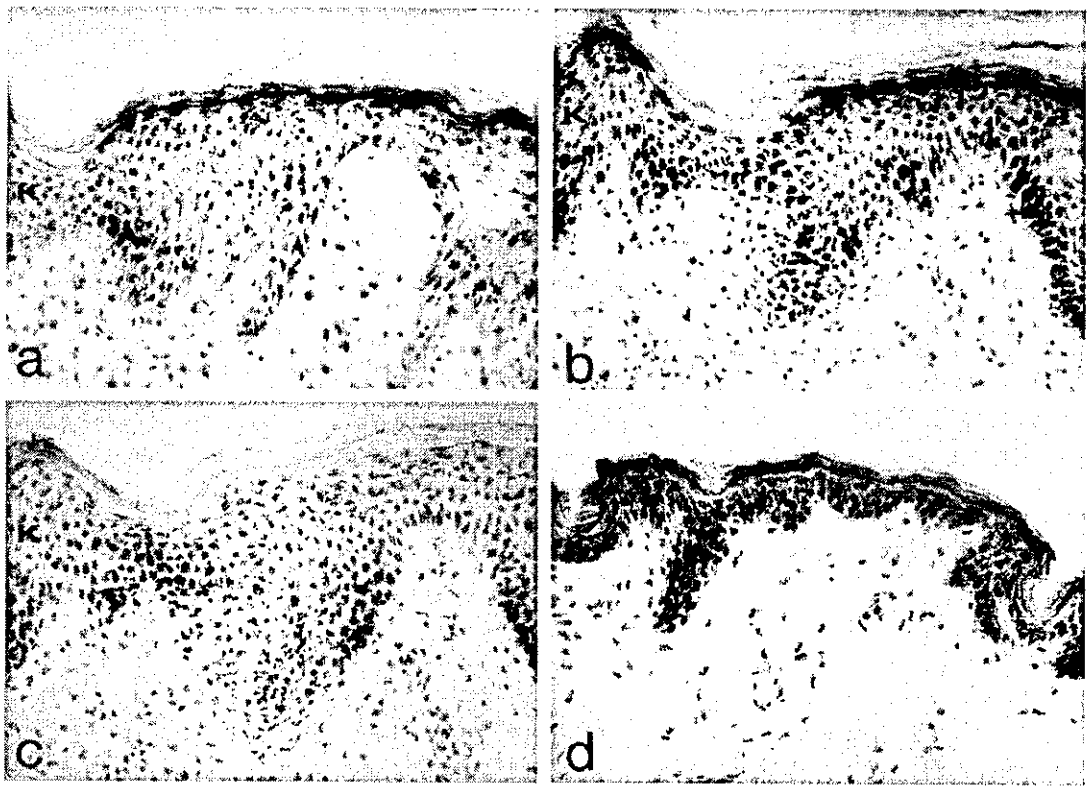

Figure 2. Immunohistochemical staining of thymus and activation-regulated chemokine (TARC) in the lesional skin of patients with bullous pemphigoid (BP). (a) Immunoreactive TARC was strongly positive in epidermal keratinocytes (KC), especially in the basal layer, in the lesional skin of patients with BP. (b) Non-bullous lesions in patients with BP also stained TARC in the epidermal KCs. (c) In contrast, TARC was virtually negative in normal skin. (d) When the isotype matched control was used, no positive staining was observed.

Discussion

In this study, we revealed that: (i) fluid TARC/CCL17 levels in patients with BP were significantly higher than those in burn patients or healthy control subjects; (ii) serum TARC levels in patients with BP were significantly higher than those in PV patients or healthy control subjects; (iii) serum TARC levels in patients with BP significantly decreased after treatment with oral corticosteroid treatment; (iv) serum TARC levels significantly correlated with the eosinophil numbers in peripheral blood; (v) immunoreactive TARC was detected in the epidermal KCs in the lesional and normal-appearing skin of patients with BP; (vi) both CCR4+ CD4+ T cells and CXCR3+ CD4+ cells were observed in the lesional skin; and (vii) CCR4 positivity in CD4+ CD45RO+ cells of PBMCs in patients with BP was higher than that in healthy control subjects, whereas there was no significant difference of CXCR3 positivity in CD4+ CD45RO+ cells of PBMCs between patients with BP and healthy control subjects.

BP is a subepidermal blistering disease associated with autoantibodies that recognize hemidesmosomal proteins. It was observed previously by immunohistochemistry and by *in situ* hybridization that IL-4, IL-5 and IL-13 are expressed in the lesional skin of patients with BP.[13] Moreover, Budinger *et al.*[7] reported that autoreactive T cells, which participate in the blister formation of BP and recognize the extracellular domains of BPAG2, show the characteristics of Th2. However, very recently, Lin *et al.*[9] found that the autoreactive T cells possess not only Th2-type but also Th1-type cytokine. Thus, there is now some conjecture as to whether BP shows Th1- or Th2-type polarization.

TARC/CCL17 is a ligand for CCR4[18] and CCR8,[26] and plays an important role for the migration of these receptor-expressing cells. Previously, it was reported from *in vitro* studies, that Pam212 cells, a murine