

Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis

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Background: T_H2 cells and eosinophils selectively express CC chemokine receptor 4 and CCR3, respectively, and their chemokine ligands are likely to play important roles in the pathogenesis of atopic dermatitis (AD).

Objective: The purpose of this study was to demonstrate the presence of thymus and activation-regulated chemokine (TARC) in platelets and its release during clotting and to evaluate the circulating levels of TARC, macrophage-derived chemokine (MDC), and eotaxin in control subjects and patients with AD.

Methods: We compared plasma and serum contents of TARC, MDC, and eotaxin. We measured TARC contents in platelet lysates. We analyzed the correlation of plasma levels of TARC, MDC, and eotaxin with various clinicolaboratory parameters in patients with AD.

Results: Serum contents of TARC rapidly increased during clot-

ting, whereas those of MDC and eotaxin increased only slightly. We demonstrated that platelets contained TARC, and its levels were dramatically elevated in patients with AD. Platelets also released TARC on stimulation with thrombin. We therefore evaluated circulating levels of these chemokines in control subjects and patients with AD by using plasma samples. Plasma TARC levels were significantly increased in patients with AD ($P < .0001$) and showed significant correlations with severity scoring of atopic dermatitis (SCORAD) index ($r = 0.665$, $P < .00001$), serum lactate dehydrogenase levels ($r = 0.696$, $P = .00001$), eosinophil counts ($r = 0.381$, $P = .007$), and platelet counts ($r = 0.562$, $P < .0001$). Similarly, plasma MDC levels were significantly increased in patients with AD ($P < .0001$) and showed significant correlations with SCORAD index ($r = 0.727$, $P < .0001$), serum lactate dehydrogenase levels ($r = 0.861$, $P < .0001$), eosinophil counts ($r = 0.505$, $P = .005$), and platelet counts ($r = 0.370$, $P = .01$). On treatment, plasma TARC and MDC levels were dramatically decreased in accordance with improved SCORAD scores ($P = .0012$ and $P = .0007$, respectively). On the other hand, plasma eotaxin levels did not show any significant increase or correlation with any of the clinical parameters in patients with AD.

Conclusion: Platelets from patients with AD contain high levels of TARC. Thus platelets might play an important role in AD pathogenesis by releasing T_H2-attracting TARC on activation. Furthermore, circulating levels of TARC and MDC, but not those of eotaxin, correlate well with the disease activity of AD. (*J Allergy Clin Immunol* 2002;110:139-46.)

Key words: Atopic dermatitis, chemokine, chemokine receptor, platelet, T_H2, eosinophil

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Atopic dermatitis (AD) is a chronic inflammatory disease of the skin that is frequently associated with high serum IgE levels and eosinophilia.¹ Acute lesions

Abbreviations used

AD:	Atopic dermatitis
CLA:	Cutaneous lymphocyte antigen
LDH:	Lactate dehydrogenase
MDC:	Macrophage-derived chemokine
SCORAD:	Severity scoring of atopic dermatitis
TARC:	Thymus and activation-regulated chemokine

are characterized by marked perivascular infiltration of CD4⁺ memory-effector T cells expressing high levels of cutaneous lymphocyte antigen (CLA).² Memory-effector T cells of the CD4 lineage are now subdivided into T_H1 and T_H2 types in accordance with their cytokine profiles. T_H1 cells produce IL-2, IFN- γ , and TNF- α , for example, and are responsible for cell-mediated immunity, whereas T_H2 cells produce IL-4, IL-5, IL-6, and IL-13, for example, and are involved in humoral immunity and allergic diseases.³ T_H2-dominant immune responses to environmental allergens in the skin on the basis of undefined genetic predispositions are the central features of AD.²

Chemokines are a group of cytokines that regulate migration and activation of various types of leukocytes through a group of 7 transmembrane G protein-coupled receptors.⁴ Recent studies have revealed that T_H1 and T_H2 cells differentially express chemokine receptors. T_H1 cells selectively express CXCR3 and CCR5, whereas T_H2 cells express CCR4 and, less frequently, CCR3.⁴ Furthermore, essentially all CLA⁺ skin-seeking memory-effector T cells were found to express CCR4.^{5,6} On the other hand, eosinophils mainly express CCR3.^{7,8} Thus the CCR4 ligands, as well as those of CCR3, are likely to play important roles in the pathogenesis of AD through selective recruitment of T_H2 cells and eosinophils into inflamed skin.

CCR4 is the receptor for 2 CC chemokines, thymus and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22, whereas CCR3 is the receptor for eotaxin/CCL11 and many other CC chemokines known to act on eosinophils.^{4,8} Increased expression of TARC and MDC in the skin was demonstrated in the NC/Nga strain of mouse, a murine model of AD.⁹ Serum levels of TARC and MDC were shown to be increased in patients with AD but not in patients with psoriasis and other T_H1-type diseases.^{10,11} Enhanced infiltration of CCR4-expressing T cells in AD skin lesions was also demonstrated.¹² Thus it is likely that TARC and MDC are produced in large quantities in AD lesional skin, leading to skin infiltration of T cells expressing CCR4 and to increased levels of these chemokines in the circulation.

The use of serum samples in the previous studies for the evaluation of circulating levels of TARC and MDC,^{10,11} however, might have potential problems

because (1) some chemokines are known to be secreted from platelets,¹³ (2) many chemokines can be released from Duffy antigen receptor for chemokines (ie, the chemokine scavenger receptor expressed on erythrocytes),^{14,15} and (3) some chemokines are even adsorbed to newly formed clotting matrix.¹⁶ Here we compared the contents of TARC, MDC, and eotaxin in plasma and serum samples simultaneously obtained from the same donors. We found that platelets contained TARC and released it during clotting. We therefore reevaluated the circulating levels of TARC, MDC, and eotaxin in control subjects and patients with AD using plasma samples.

METHODS

Subjects and samples

A total of 29 patients with AD (22 children <16 years of age and 7 adults ≥ 16 years) and 29 healthy age-matched donors (19 children <16 years of age and 10 adults ≥ 16 years) were examined. Mean age $\pm$ SD was 8.0 ± 5.0 in children with AD, 9.9 ± 3.8 in control children, 24.6 ± 4.7 in adults with AD, and 27.8 ± 3.8 in control adults. The healthy control subjects had no history of allergic diseases, and their serum IgE levels were less than 160 IU/mL, with no detectable specific IgE antibodies to common inhaled allergens, as determined with Phadiatope (Pharmacia Upjohn, Uppsala, Sweden). The patients with AD met the clinical diagnostic criteria for the disease¹; that is, they showed high serum IgE levels and were sensitive to more than one inhaled or food allergen, as demonstrated by a positive RAST result. Severity of AD was evaluated by using the severity scoring of atopic dermatitis (SCORAD) index.¹⁷ Serum IgE and lactate dehydrogenase (LDH) levels and peripheral blood eosinophil and platelet counts were routinely determined. Blood samples were obtained on 35 occasions from 22 children with AD and on 18 occasions from 7 adults with AD in the course of treatment for AD. For control donors, blood sampling was done once for each individual. Venous blood was collected in EDTA-containing tubes to prepare plasma, and plasma was separated within 30 minutes by means of centrifugation at 1800g and 4°C for 10 minutes twice and stored at -20°C until assay. Venous blood was taken in Vacutainer tubes containing SST gel and clot activator (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1800g and 4°C for 10 minutes after incubation and for 1 hour at room temperature, unless otherwise indicated, to prepare serum. Plasma chemokine levels were also determined before and after treatment for 15 patients with severe AD (SCORAD >70). After the first sampling of blood, the patients were treated with topical corticosteroids with high to medium potency, skin hydration with bathing, application of an occlusive agent (eg, petroleum jelly after bathing), and oral antihistamines (eg, diphenhydramine and ketotifen). None of the patients received systemic steroids. After 1 to 2 weeks of treatment, the patients were evaluated for disease severity, and the second blood sampling was performed. This study was carried out with the approval of the Ethics Committee of National Mie Hospital. Informed consent was obtained from all subjects.

ELISA for chemokines

Eotaxin and MDC levels were determined by using the sandwich-type ELISA, as described previously.^{10,15} A sandwich-type ELISA for TARC with a detection limit of 0.6 pg/mL was developed by using 2 newly generated mouse monoclonal anti-TARC antibodies and will be described elsewhere (Morita et al, in preparation). A sandwich-type ELISA for RANTES with a detection limit of 2 pg/mL was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Platelets

Platelets were purified from platelet-rich plasma, as previously described.¹⁸ The purity of platelets was routinely greater than 99%. Purified platelets were mechanically lysed by means of ultrasonic treatment. TARC and RANTES contents in platelet lysates were measured with ELISA, and total protein contents were measured with a protein assay kit (Bio-Rad Laboratories, Hercules, Calif). Stimulation of platelets with thrombin was also carried out, as previously described.¹⁸ Platelets at $1 \times 10^8/\text{mL}$ were incubated at 37°C for 60 minutes with 1 U/mL thrombin (Sigma, St Louis, Mo). TARC contents in the supernatants were measured with ELISA.

Statistical analysis

Data on serum IgE levels, blood eosinophil counts, and plasma contents of TARC, MDC, and eotaxin were expressed as geometric means because logarithmically transformed values of the data followed normal distribution. Differences were analyzed with the Mann-Whitney *U* test for unpaired samples and with the Wilcoxon signed-rank test for paired samples. Spearman correlation coefficients between 2 parameters were calculated.

RESULTS

Rapid increases in serum TARC/CCL17 levels during blood clotting

We simultaneously collected plasma and serum samples from control subjects and patients with AD and determined the contents of TARC/CCL17, MDC/CCL22, and eotaxin/CCL11. As shown in Fig 1, TARC contents were dramatically increased in the serum samples. Furthermore, the serum/plasma ratio was much greater in patients with AD than in control subjects (16.2 ± 9.2 [$n = 14$] vs 6.0 ± 5.0 [$n = 21$], respectively; $P < .01$). On the other hand, serum MDC contents were only slightly increased from those in plasma samples, and a significant increase was only seen in control subjects. In the case of eotaxin, there were also slight but significant increases in serum contents in both control subjects and patients with AD.

We next determined the time course of serum chemokine levels by collecting serum samples at various time points during the clotting reaction at 37°C . Blood samples were obtained from 6 patients with AD. TARC contents rapidly increased during the clotting reaction, reaching maximal levels at 10 minutes (data not shown). On the other hand, MDC contents showed small increases, and those of eotaxin increased gradually during the 2 hours of incubation (data not shown). The latter was most probably caused by the release of eotaxin from Duffy antigen receptor for chemokines, the chemokine scavenger receptors expressed on erythrocytes,¹⁴ during incubation, as described previously.¹⁵ Consistent with this idea, we observed release of large amounts of eotaxin by washing erythrocytes at 4°C with a citrate buffer, pH 3.0 (data not shown).

Elevated contents of TARC/CCL17 in platelets from patients with AD

The rapid increases in serum TARC contents during clotting were likely caused by release of TARC from platelets. Furthermore, patients with AD generally

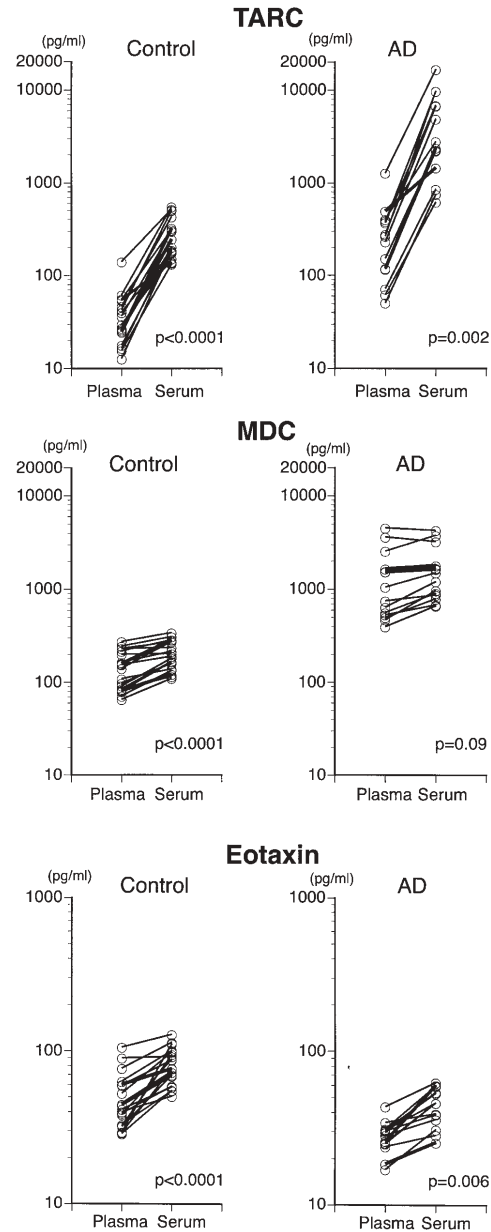


FIG 1. Comparison between plasma and serum for contents of TARC, MDC, and eotaxin. Plasma and serum samples were simultaneously obtained from control subjects ($n = 21$) and patients with AD ($n = 14$). Amounts of TARC, MDC, and eotaxin were measured with ELISA. All measurements were done in triplicate, and mean values were obtained. The mean values of TARC were as follows: control subjects, 35.2 pg/mL in plasma (range of SD, 19.3–64.1 pg/mL) and 240.5 pg/mL in serum (range of SD, 150.8–383.3 pg/mL); patients with AD, 253.2 pg/mL in plasma (range of SD, 80.3–793.4 pg/mL) and 3225.9 pg/mL in serum (range of SD, 1174.7–8859.0 pg/mL). The mean values of MDC were as follows: control subjects, 132.3 pg/mL in plasma (range of SD, 82.1–213.0 pg/mL) and 195.1 pg/mL in serum (range of SD, 137.1–277.6 pg/mL); patients with AD, 1158.3 pg/mL in plasma (range of SD, 505.1–2656.5 pg/mL) and 1590.9 pg/mL in serum (range of SD, 754.9–3352.7 pg/mL). The mean values of eotaxin were as follows: control subjects, 45.9 pg/mL in plasma (range of SD, 31.1–67.8 pg/mL) and 79.1 pg/mL in serum (range of SD, 60.1–103.9 pg/mL); patients with AD, 29.8 pg/mL in plasma (range of SD, 20.4–43.6 pg/mL) and 44.1 pg/mL in serum (range of SD, 33.2–58.5 pg/mL).

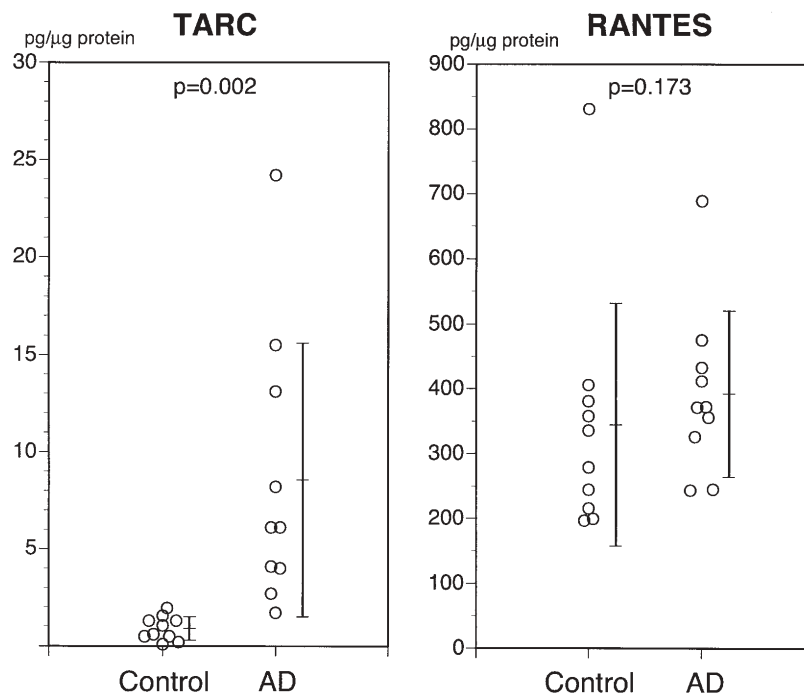


FIG 2. Presence of TARC in platelets. Highly purified platelets were obtained from control subjects ($n = 10$) and patients with AD ($n = 10$). Contents of TARC and RANTES in platelet lysates were measured with ELISA. All measurements were done in triplicate, and mean values were obtained.

showed much steeper increases in serum TARC contents than control subjects (Fig 1). We, however, found no significant difference in blood platelet counts between control subjects and patients with AD ($30.7 \pm 5.2 \times 10^4/\text{mm}^3$ vs $35.8 \pm 9.8 \times 10^4/\text{mm}^3$, respectively). We therefore determined TARC contents in platelet lysates prepared from 10 control subjects and 10 patients with AD. As shown in Fig 2, platelets indeed contained TARC. Furthermore, platelets from patients with AD consistently contained much larger amounts of TARC than those from control subjects (mean $\pm$ SD values of control subjects and patients with AD: 0.9 ± 0.6 pg/ μ g protein and 8.6 ± 7.1 pg/ μ g protein, respectively; $P = .002$). On the other hand, no such significant difference was seen in the platelet contents of RANTES/CCL5 between control subjects and patients with AD (mean $\pm$ SD values: 344.9 ± 187.2 pg/ μ g protein and 392.4 ± 127.9 pg/ μ g protein, respectively; $P = .173$). To further confirm the biologic significance of TARC in platelets, we treated platelets with 1 U/mL thrombin. Platelets released $79.2\% \pm 3.6\%$ of TARC on thrombin stimulation, whereas spontaneous release was $32.7\% \pm 10.7\%$ ($n = 6$).

Elevated plasma levels of TARC and MDC in patients with AD

The observed differences between serum and plasma in terms of chemokine contents prompted us to reevaluate the circulating levels of TARC, MDC, and eotaxin in

control subjects and patients with AD by using their plasma samples. As shown in Fig 3, the plasma levels of TARC and MDC were significantly elevated in patients with AD ($P < .0001$). On the other hand, there were no significant differences in the plasma eotaxin levels between patients with AD and control subjects ($P = .121$). Notably, there were significant age-related changes in the plasma MDC levels among the control subjects; those younger than 16 years ($n = 19$) and those 16 years of age or older ($n = 10$) had mean MDC values of 443.2 pg/mL (range of SD, 219.4-896.5 pg/mL) and 121.6 pg/mL (range of SD, 69.5-212.7 pg/mL), respectively ($P = .002$). No such age-related changes were seen in the plasma levels of TARC and eotaxin among the control subjects.

Correlation of plasma TARC and MDC levels with other disease parameters in patients with AD

We next analyzed the correlation of the plasma chemokine level and SCORAD index in the patients with AD ($n = 53$; samples obtained from the same donors in different occasions were included). As shown in Fig 4, A, there were strong positive correlations between SCORAD scores and plasma levels of TARC ($r = 0.665$, $P < .0001$) and MDC ($r = 0.727$, $P < .0001$). There was, however, no significant correlation between SCORAD scores and

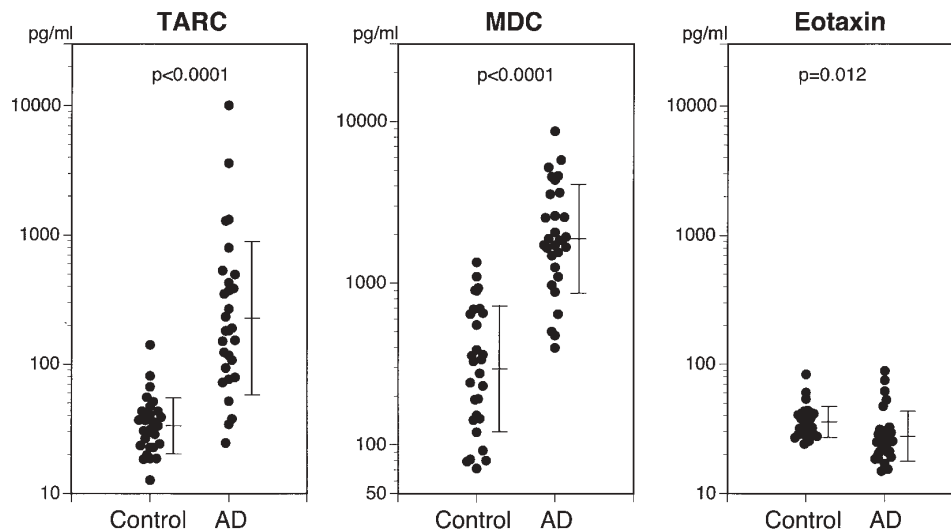


FIG 3. Elevated plasma levels of TARC and MDC in patients with AD. TARC, MDC, and eotaxin contents in plasma samples obtained from control subjects and patients with AD were measured with ELISA. All measurements were done in triplicate, and mean values were obtained. The mean values of control subjects and patients with AD were as follows: TARC, 33.4 pg/mL (range of SD, 20.2-55.0 pg/mL) and 228.6 pg/mL (range of SD, 58.2-897.2 pg/mL), respectively; MDC, 296.7 pg/mL (range of SD, 121.5-724.4 pg/mL) and 1891.2 pg/mL (range of SD, 869.9-4111.3 pg/mL), respectively; and eotaxin, 36.0 pg/mL (range of SD, 27.3-47.3 pg/mL) and 27.9 pg/mL (range of SD, 17.9-43.7 pg/mL), respectively.

plasma eotaxin levels ($r = 0.104$, $P = .483$). We also compared the plasma chemokine levels before and after treatment. Fifteen patients with severe disease (>70 SCORAD scores) were treated with a standard combination of skin hydration, topical steroids, and oral antihistamines. After 1 to 2 weeks, when their symptoms were improved, the second plasma samples were obtained. As shown in Fig 4, B, the mean SCORAD index was decreased from 81.7 to 39.3 ($P = .0007$). Accordingly, the plasma levels of TARC and MDC were significantly decreased ($P = .0012$ and $P = .0007$, respectively). On the other hand, no such significant change was seen in the plasma eotaxin levels ($P = .3$).

We further analyzed the correlation between the plasma chemokine levels and various clinicolaboratory parameters known to be increased in patients with AD. The results are summarized in Table I. Plasma TARC and MDC levels strongly correlated with serum LDH levels and weakly with blood eosinophil counts. Notably, plasma TARC and MDC levels also significantly correlated with blood platelet counts. Plasma TARC and MDC levels, however, showed no significant correlation with total serum IgE levels. Plasma eotaxin levels showed no significant correlation with any of these parameters.

DISCUSSION

Platelets play an important role not only in blood coagulation but also in inflammation.^{19,20} Involvement of platelets in the pathogenesis of allergen-induced asthma

has been reported.^{21,22} Selective depletion of circulating platelets was shown to reduce the late-phase response, eosinophil infiltration, and bronchial hyperresponsiveness in animal models of asthma.^{23,24} Platelets are also known to release various chemokines.²⁵⁻²⁷ Release of RANTES from platelets on cross-linking of the high-affinity receptor for IgE has been suggested to promote allergic reactions by attracting CCR3-expressing cells, such as eosinophils.¹⁸ Here we have demonstrated, for the first time, that platelets contain TARC (Fig 2) and release it on coagulation and thrombin stimulation. Furthermore, the contents of TARC, but not those of RANTES, in platelets are dramatically elevated in patients with AD (Fig 2). Plasma contents of TARC also strongly correlated with blood platelet counts in patients with AD (Table I). These results suggest that the T_H2 -dominant conditions in AD enhance production of TARC, as well as that of platelets, by megakaryocytes in the bone marrow. Platelets are also known to express CCR4 and CXCL4, and their ligands, TARC, MDC, and stromal cell-derived factor 1/CXCL12, have been shown to induce platelet aggregation through a pathway dependent on ADP and the P2Y(1)-type ADP receptor.²⁸⁻³¹ Thus release of TARC from activated platelets might further enhance their aggregation and release of TARC in an autocrine manner. Collectively, platelets in patients with AD, by releasing TARC within inflamed microvasculature, might play an important role in extravasation of T_H2 cells and CLA⁺ skin-seeking memory T cells expressing CCR4.⁴

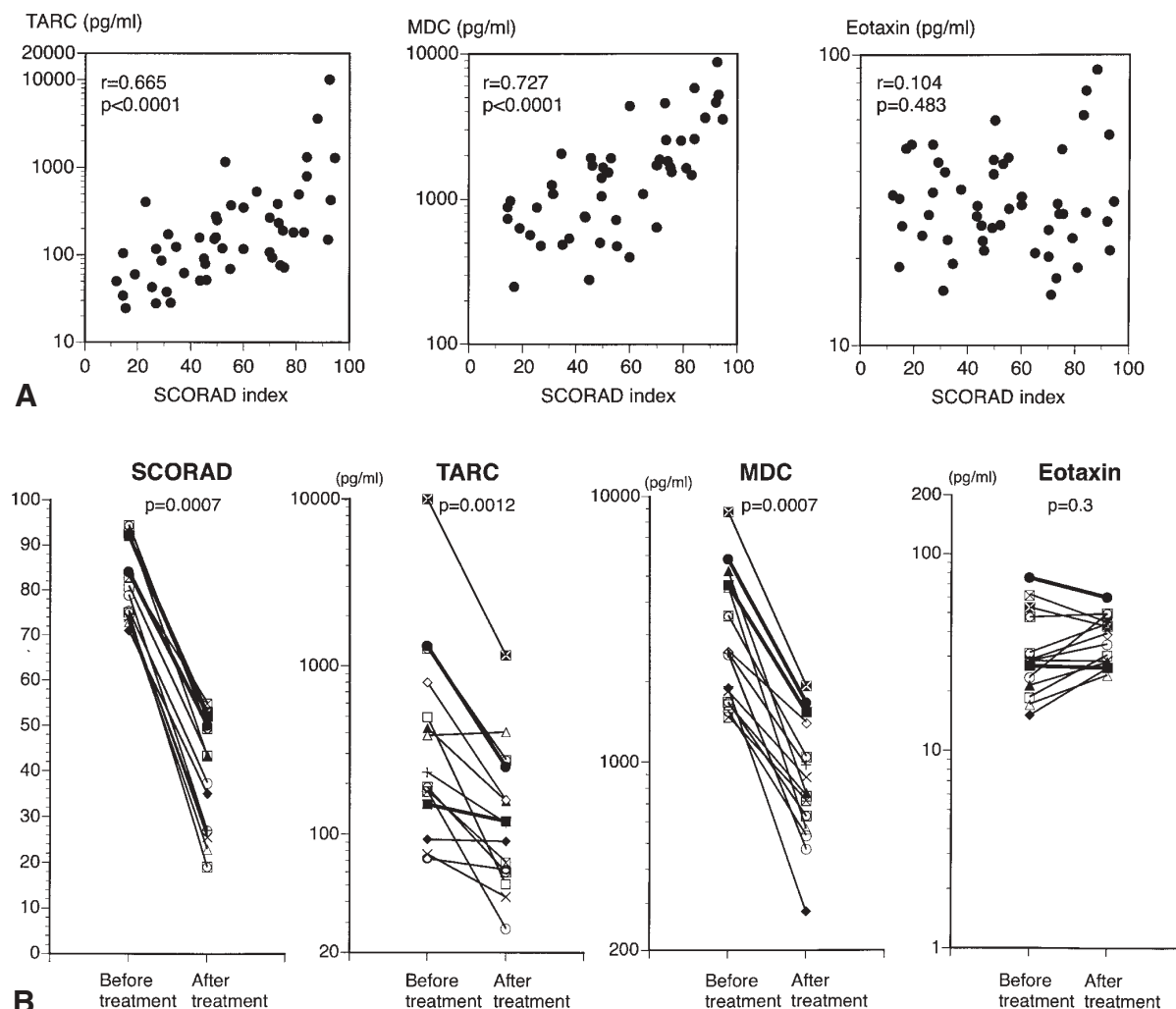


FIG 4. A, Correlation of plasma chemokine levels with disease activity of AD. Contents of TARC, MDC, and eotaxin in plasma samples obtained from patients with AD were measured with ELISA. All measurements were done in triplicate, and mean values were obtained. Disease severity was expressed by SCORAD index. B, Changes in SCORAD index and plasma chemokine levels before and after treatment. Mean values before and after treatment were as follows: SCORAD score, 81.7 and 39.3, respectively; TARC, 346.7 pg/mL (range of SD, 93.0-1292.9 pg/mL) and 120.5 pg/mL (range of SD, 45.5-319.0 pg/mL), respectively; MDC, 2876.3 pg/mL (range of SD, 1644.6-5030.6 pg/mL) and 836.8 pg/mL (range of SD, 494.0-1417.4 pg/mL), respectively; eotaxin, 30.3 pg/mL (range of SD, 18.6-49.5 pg/mL) and 36.1 pg/mL (range of SD, 27.0-48.3 pg/mL), respectively.

TABLE I. Spearman correlation coefficients between plasma chemokine levels and other laboratory parameters in patients with AD

	LDH	Eosinophil count	Platelet count	IgE level
TARC	0.696 ($P < .0001$)	0.381 ($P = .007$)	0.562 ($P < .0001$)	0.004 ($P = .835$)
MDC	0.861 ($P < .0001$)	0.505 ($P = .0002$)	0.370 ($P = .01$)	0.008 ($P = .635$)
Eotaxin	0.003 ($P = .858$)	-0.260 ($P = .07$)	0.036 ($P = .819$)	0.008 ($P = .635$)

A total of 48 plasma samples were analyzed.

Previous reports demonstrated dramatic increases in serum TARC and MDC contents in patients with AD.^{10,11} However, TARC contents in the serum are highly increased because of its release from platelets (Fig 1).

Therefore we reevaluated the circulating contents of TARC, MDC, and eotaxin in control subjects and patients with AD by using plasma samples. We demonstrated that TARC and MDC levels in plasma were

increased in patients with AD and highly correlated with disease activity and the clinical course (Figs 3 and 4). This is the first observation that not only TARC but also MDC levels in circulation closely correlate with clinical status of AD. Correlation coefficients of plasma MDC levels with SCORAD scores, serum LDH levels, and eosinophil counts were even higher than those of plasma TARC levels (Fig 4 and Table I). Furthermore, there were only small differences between serum and plasma contents of MDC (Fig 1). Thus MDC might be more useful to monitor the disease course of AD than TARC. Notably, however, there are striking age-related differences in the plasma MDC levels among control subjects: Those younger than 16 years have much higher levels than those 16 years of age or older. Further studies are necessary to determine the age-related normal levels of MDC and a possible role of MDC during development.

In contrast, plasma levels of eotaxin were not significantly increased in patients with AD (Fig 3). Thus CCR3-directed chemokines different from eotaxin, such as RANTES, might play major roles in the accumulation of eosinophils in the lesional skin of patients with AD.^{32,33} However, Hossny et al³⁴ recently reported that plasma eotaxin levels were significantly elevated in patients with AD. We do not know the cause of the discrepancy, but possible explanations might be different patient backgrounds, different timing of sampling, and different methods of assay. Furthermore, our observation does not exclude the possible involvement of eotaxin in the pathogenesis of AD. In fact, eotaxin expression was demonstrated to be highly upregulated in AD chronic skin lesions but not in acute skin lesion.³⁵

In conclusion, TARC and MDC, the T_H2 -type chemokines acting on CCR4, are likely to be closely involved in the pathogenesis of AD and possibly other T_H2 -type diseases. Monitoring circulating levels of TARC and MDC might thus serve as useful markers for disease conditions in AD. Furthermore, platelets might play important roles in AD pathogenesis and other T_H2 -type diseases by releasing TARC.

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