

Levels of soluble intercellular adhesion molecule-1, soluble E-selectin, tumor necrosis factor- α , and soluble tumor necrosis factor receptor p55 and p75 in atopic children

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During inflammation, membrane expression of adhesion molecules and tumor necrosis factor (TNF)-receptors (TNF-R) are increased, and soluble forms of these molecules are released. This study analyzed plasma levels of sICAM-1 and sE-selectin as well as TNF- α , sTNF-R55, and sTNF-R75 in nonallergic (NAA) and allergic asthma patients (AA), atopic dermatitis patients (AD), and healthy children (HC) by ELISA. Plasma levels of sICAM-1, sE-selectin, and sTNF-R, but not TNF- α , were detectable, but were not significantly different between the patient groups and healthy children. In the AA group, a significant correlation ($r_s=0.78$, $P=0.008$) was found between sICAM-1 and sE-selectin levels. Furthermore, a significant correlation was found between sTNF-R55 and sTNF-R75 levels in the AA group ($r_s=0.70$, $P=0.025$) and in the AD group ($r_s=0.69$, $P=0.027$). In AD patients, a significant correlation was observed between sE-selectin and the disease severity, as measured by the SCORAD index ($r_s=0.73$, $P=0.038$). Our data demonstrate that plasma levels of sICAM-1, sE-selectin, TNF- α , sTNF-R55, and sTNF-R75 were not different between atopic and nonatopic children during a stable phase of the disease. In AD patients, levels of sE-selectin seemed to be related to clinical severity of the disease.

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Several cytokines, including tumor necrosis factor (TNF)- α , promote the induction and upregulation of adhesion molecules, both on the endothelium and on leukocyte surfaces (1). At sites of inflammation, increased expression of intercellular adhe-

sion molecule-1 (ICAM-1, CD54), vascular cell adhesion molecule-1 (VCAM-1, CD106), and E-selectin (CD62E) on vascular endothelium in the lung and skin have been demonstrated (2). In skin biopsies of patients with respiratory allergy, allergen-induced upregulation of E-selectin on endothelial cells was reported during the late-phase reaction after intradermal allergen (ragweed or house-dust mites) injection (3). In allergic-asthma patients, increased expression of ICAM-1 was shown on bronchial epithelial cells compared to patients with chronic bronchitis or healthy subjects (4, 5). In these patients, increased expression of both ICAM-1 and E-selectin on bronchial endothe-

Abbreviations used: AD: atopic dermatitis; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; CLA: cutaneous lymphocyte-associated antigen; TNF- α : tumor necrosis factor- α ; TNF-R: TNF-receptor; sTNF-R: soluble TNF-R; AA: allergic asthma; NAA: nonallergic asthma; HC: healthy controls; SAFT: skin application food test; ELISA: enzyme-linked immunosorbent assay; RAST: radio-allergosorbent test; RT: room temperature; BSA: bovine serum albumin; IL: interleukin; mAb: monoclonal antibody.

lium was observed (5). These results indicate that adhesion molecules are upregulated in allergic inflammation, probably through the presence of inflammatory cytokines.

The interaction between circulating T cells and microvascular endothelial cells is a critical determinant of the homing process. In the skin, interaction between the cutaneous lymphocyte-associated antigen (CLA) on skin homing T cells and E-selectin has been supposed to be involved in tissue-selective T-cell extravasation (6, 7).

During inflammation, not only is the expression of adhesion molecules increased, but also soluble adhesion molecules are released (8–10). The presence of soluble adhesion molecules in the circulation of patients with various inflammatory disease states has suggested that plasma levels may reflect disease activity. Determination of the concentration of soluble adhesion molecules could be useful in studying the process of allergic inflammation.

In patients with atopic dermatitis, but not bronchial asthma, plasma TNF- α concentrations were reported to be increased during inflammation (11). In contrast, Koizumi et al. (12) reported elevated serum TNF- α levels during bronchial asthma attacks. The activities of TNF- α are mediated via TNF-receptors (TNF-R), expressed on a number of cell types (13, 14). Two different types of TNF-R have been characterized with molecular masses of 55 kDa (TNF-R55) and of 75 kDa (TNF-R75) (15). These receptors are also present in soluble form in the circulation.

Cellular activation has been shown to result in increased presence of soluble TNF-R (sTNF-R), probably via shedding of cell-bound receptors (16). These sTNF-R have retained the ability to bind TNF- α , thereby competing with cell-bound TNF-R and neutralizing TNF- α activity. Normally, serum concentrations of the sTNF-R are rather low (17). However, in various pathologic states accompanied by immune activation, including sepsis (18–20) and autoimmune disease (21, 22), and due to reduced clearing in chronic renal failure (23), the concentrations of sTNF-R increase substantially. Hence, sTNF-R could well be released in the course of immunologic activation related to allergy. Therefore, we studied TNF- α and sTNF-R levels in several groups of atopic patients.

This study analyzed whether the above mentioned markers were different between atopic and nonatopic children and whether these levels were related to disease activity. To this end, we evaluated the levels of sICAM-1, sE-selectin, TNF- α , sTNF-R55, and sTNF-R75 in plasma samples of children with (allergic and nonallergic) asthma and atopic dermatitis, and of healthy controls.

Material and methods

Study population

We studied children suffering from atopic dermatitis (AD, $n=10$) with a mean age of 29.3 months (range 7–48 months), allergic asthma (AA, $n=9$) with a mean age of 36.6 months (range 12–54 months), nonallergic asthma (NAA, $n=9$) with a mean age of 24.3 months (range 7–56 months), and 10 healthy children (HC) with a mean age of 32.3 months (range 8–53 months). The medical ethical committee of University Hospital Rotterdam approved this study. Informed consent was obtained from the parents of the children before participation in the study.

AD patients were selected for history of mild to severe eczema according to the diagnostic criteria of Hanifin & Rajka (24). They also had a positive scratch test or skin application food test (SAFT) (25) specific for milk, egg, peanut, wheat, soybean, or codfish and a positive radioallergosorbent test (RAST) (26) specific for food mix (Pharmacia, Uppsala, Sweden) or inhalation allergens (Phadiatop CAP RAST, Pharmacia). The severity of the AD was determined by the modified SCORAD system (27), which is a composite index including the assessment of two items in a standardized way: 1) extent and 2) intensity (erythema, edema/papulation, oozing/crust, excoriation, lichenification, and dryness). This scoring system reflects the modified consensus of the European Task Force on Atopic Dermatitis. Unlike the initial version of the SCORAD index (28), subjective symptoms are not included in the scoring of AD.

Children with asthma were selected by the criteria of the American Thoracic Society: coughing and/or shortness of breath, at least three times a year for a period of 2 weeks (29). All patients had a positive family history of asthma and/or atopy. Asthma patients were divided into two groups, allergic and nonallergic, as judged by the presence of a positive or negative RAST (Phadiatop and food mix), respectively. All asthma patients were studied during a stable phase of their disease, and there was no need for treatment with (inhalation-) corticosteroids.

The healthy controls had no history of allergy and a negative family history as judged by a questionnaire. All healthy children lacked specific IgE for inhalation and/or food allergens as measured by RAST (Phadiatop and food mix).

Collection of plasma samples

A volume of 5 ml of heparinized peripheral blood was collected by venipuncture of each subject.

After Ficoll centrifugation, plasma samples were obtained and stored at -70°C until analysis. The analyses of sICAM-1, sE-selectin, sTNF-R55, and sTNF-R75 were performed simultaneously in plasma diluted 1:1 in sterile 10 mM phosphate-buffered saline (PBS, pH 7.2).

Determination of sICAM-1, sE-selectin, sTNF-R55, and sTNF-R75 by ELISA

sICAM-1, sE-selectin, sTNF-R55, and sTNF-R75 were measured by enzyme-linked immunosorbent assay (ELISA), as previously described (30–32). Briefly, 96-well microtiter plates (Nunc Immuno Plate Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with monoclonal antibodies (mAb) to sICAM-1 (HM.2, $3\text{ }\mu\text{g/ml}$), sE-selectin (ENA1, $10\text{ }\mu\text{g/ml}$), sTNF-R55 (MR1.1, $5\text{ }\mu\text{g/ml}$), or sTNF-R75 (MR2.2, $5\text{ }\mu\text{g/ml}$) in PBS followed by saturation with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA). Standard or diluted samples were added at $100\text{ }\mu\text{l/well}$. After 2 h at room temperature (RT), the plates were washed and incubated for 1 h at RT with biotin-labeled anti-ICAM mAb (HM.1), anti-E-selectin mAb (ENA2), or biotinylated rabbit anti-sTNF-R55/R75 immunoglobulin (Ig)G. After washing, streptavidin-conjugated peroxidase (Dako, Glostrup, Denmark) was added. Peroxidase activity was determined by addition of TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate (Kirkegaard & Perry Laboratory, Gaithersburg, MD, USA). The reaction was stopped with H_2SO_4 and read at 450 nm. The concentrations of sICAM-1, sE-selectin, sTNF-R55, and sTNF-R75 in the samples were determined by interpolation from the standard curve. The detection limits of the individual ELISA assays were 400 pg/ml for sICAM-1, 200 pg/ml for sE-selectin, 12 pg/ml for sTNF-R55, and 25 pg/ml for sTNF-R75. All monoclonal antibodies used in the different ELISAs were the kind gift of Dr W. A. Buurman.

Sandwich ELISA for detection of TNF- α in plasma

Determination of TNF- α levels in plasma of patients and healthy children was performed by a sandwich ELISA, as described previously (33). Briefly, 96-well microtiter plates (Falcon 3912, Becton Dickinson, Mountain View, CA, USA) were coated overnight at 4°C with $100\text{ }\mu\text{l/well}$ of $2.5\text{ }\mu\text{g/ml}$ mAb 61E71 in PBS. Nonspecific binding was blocked by incubating the plates with $125\text{ }\mu\text{l/well}$ PBS containing 1% BSA (Sigma) for 1 h at RT. TNF- α standard was diluted in plasma of a suitable donor. To this end, several donors were tested, and proper plasma samples were obtained.

Plasma samples (1 : 1 diluted in PBS) and rhTNF standard ($0.01\text{--}10\text{ ng/ml}$; standard plasma diluted 1 : 1 in PBS) were added in duplicate ($100\text{ }\mu\text{l/well}$) and incubated for 2 h at RT. Next, the plates were incubated for 1 h at RT with rabbit anti-TNF diluted in PBS containing 0.1% BSA, followed by 1 h incubation with polyclonal swine anti-rabbit IgG conjugated to horseradish peroxidase. After washing of the plates five times with bidest containing 0.1% Tween 20, bound enzyme activity was measured with 1 mg/ml 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS; Sigma) in a 1 : 1 mixture of 0.1 M citric acid and 0.2 M $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$, containing 0.003% H_2O_2 as substrate. The optical density was read at 414 nm. The lower detection limit of the ELISA was 40 pg/ml .

Statistical analyses

Statistical analyses were performed with STATATM (Computing Resource Center, Los Angeles, CA, USA). Statistical significance was analyzed by the Mann–Whitney test, and correlation coefficients were calculated by linear regression or the Spearman sign rank correlation. *P* values smaller than 0.05 were considered significant.

Results

Plasma levels of sICAM-1, sE-selectin, TNF- α , sTNF-R55, and sTNF-R75 in the various patient groups

We determined whether the plasma levels of sICAM-1, sE-selectin, TNF- α , sTNF-R55, and sTNF-R75 were different between atopic and non-atopic children. Table 1 summarizes the plasma levels of sICAM-1, sE-selectin, sTNF-R55, or sTNF-R75 for each patient group. No significant differences could be observed between healthy children and the various patient groups for any of the molecules analyzed. TNF- α could not be detected in any of the samples.

Relationship between plasma levels of sICAM-1, sE-selectin, sTNF-R55, or sTNF-R75 and age of the children

The plasma levels of sICAM-1, sE-selectin, sTNF-R55, and sTNF-R75 were ordered in each patient group according to age, as illustrated in Fig. 1, to examine whether age-related changes could be observed. As the age distribution in the patient groups were similar, the data obtained of the measured markers could be compared between the different groups. The data were analyzed by regres-

Table 1. Plasma levels of sICAM-1, sE-selectin, sTNF-R55, and sTNF-R75 in various patient groups

Patient group	sICAM-1 (ng/ml)	sE-selectin (ng/ml)	sTNF-R55 (pg/ml)	sTNF-R75 (pg/ml)
Healthy children	65.4* (43.2–101.8)	5.1 (2.4–15.5)	385 (230–870)	1620 (930–2930)
Nonallergic asthma	60.4 (49.4–85.7)	5.2 (3.4–8.5)	390 (350–520)	1370 (350–1820)
Allergic asthma	65.8 (50.6–126.4)	4.5 (2.4–15.5)	390 (270–1290)	860 (290–3150)
Atopic dermatitis	61.9 (56.0–76.8)	6.1 (2.6–8.5)	415 (220–760)	1160 (480–2340)

* Results are expressed as median concentrations (range). All samples were assayed in duplicate.

sion analysis. We evaluated whether the regression lines in each group showed a comparable trend with age. An age-related effect was observed for sTNF-R55 ($P=0.003$, Fig. 1A) and for sTNF-R75 ($P<0.001$, Fig. 1B). No significant age-related effects were observed for sICAM-1 (Fig. 1C) and sE-selectin (Fig. 1D).

Relationship between plasma levels of sICAM-1 and sE-selectin or sTNF-R55 and sTNF-R75

We analyzed whether a correlation was present between sICAM-1 and sE-selectin, or between sTNF-R55 and sTNF-R75 in each patient group. As shown in Figs. 2A and B, there was a significant

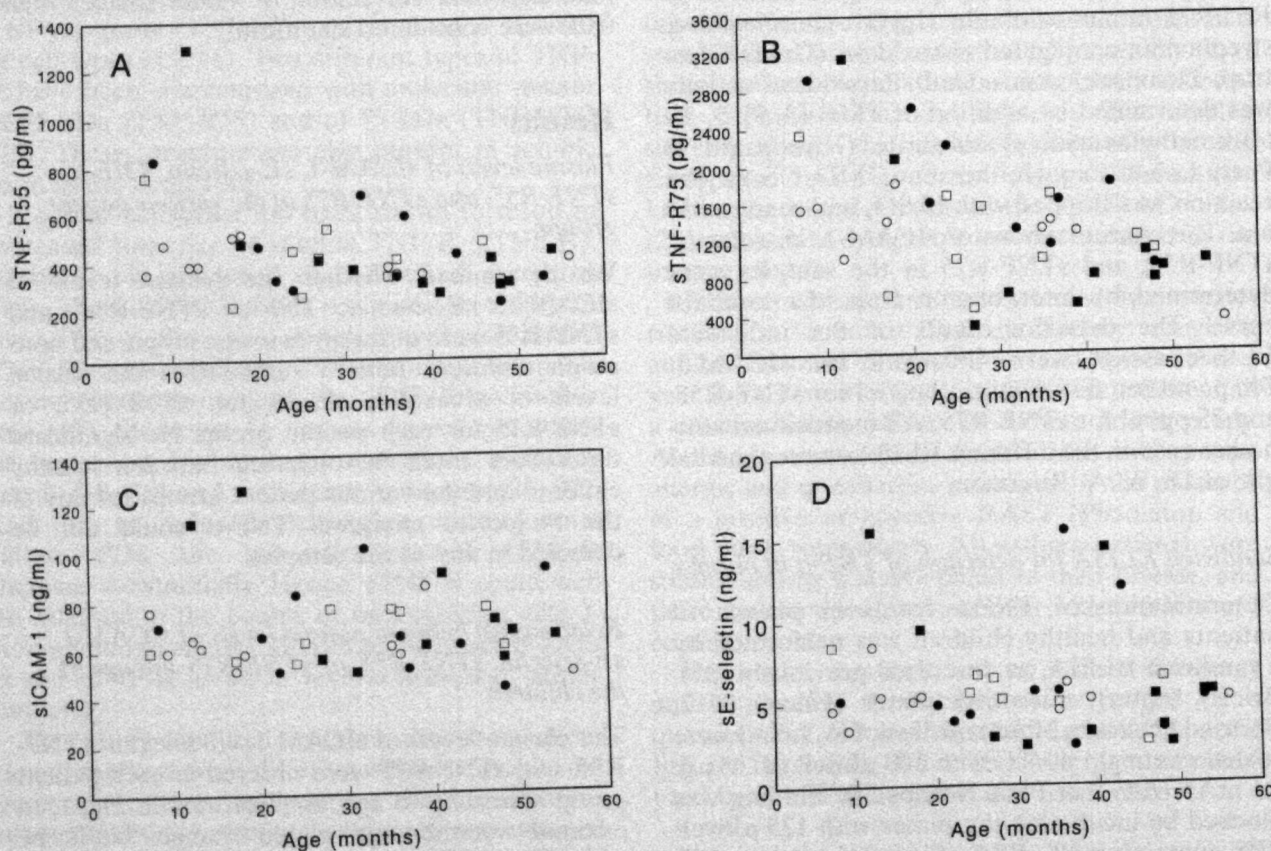


Fig. 1. A) Plasma levels of sTNF-R55 (pg/ml), B) sTNF-R75 (pg/ml), C) sICAM-1 (ng/ml), D) sE-selectin (ng/ml), in relation to age (months) of children. Healthy children are represented by closed circles, children with nonallergic asthma by open circles, children with allergic asthma by closed squares, and children with atopic dermatitis by open squares. Levels of sTNF-R55 ($P=0.003$) and sTNF-R75 ($P<0.001$) decreased significantly with age of children.

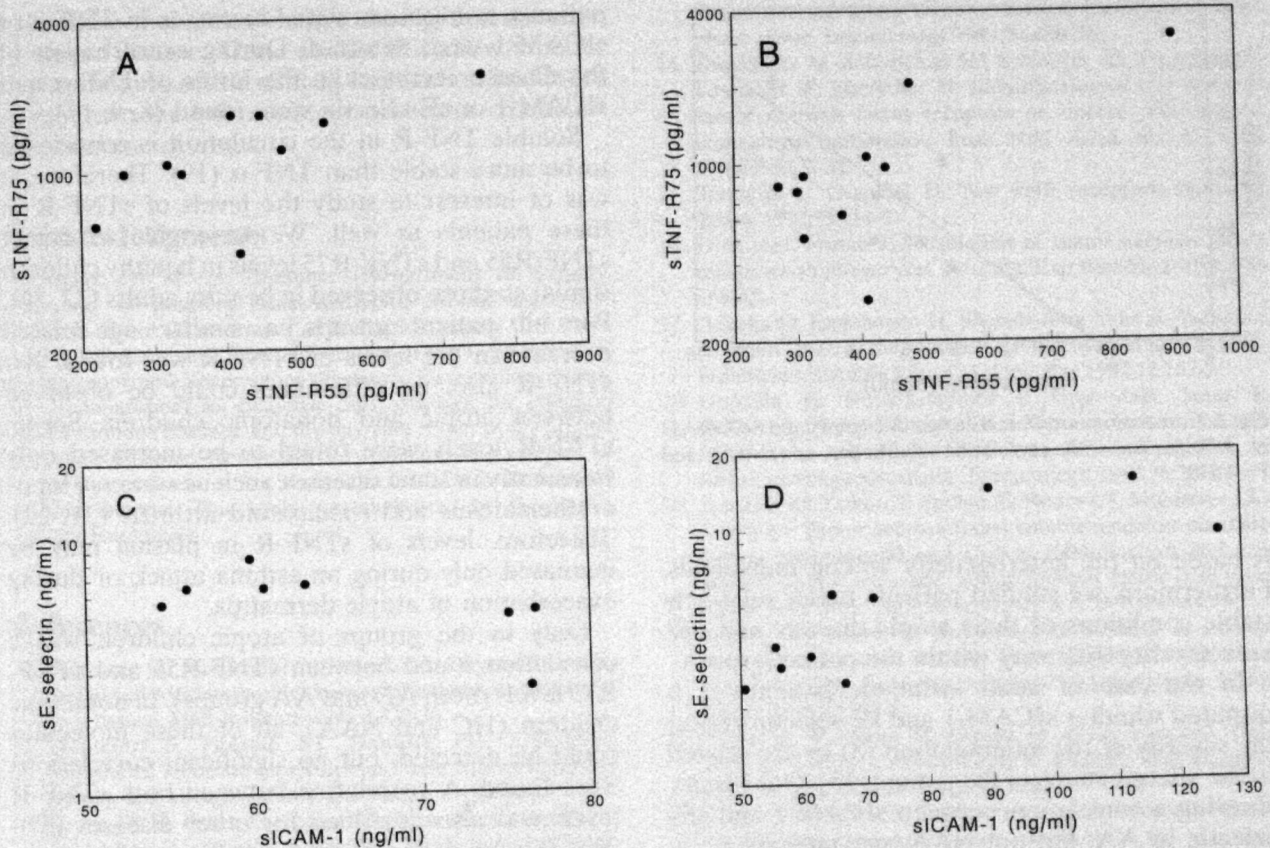


Fig. 2. Correlation between sTNF-R55 (pg/ml) and sTNF-R75 (pg/ml) or sICAM-1 (ng/ml) and sE-selectin (ng/ml) in AD and AA. A) Correlation between sTNF-R55 and sTNF-R75 in AD ($r_s=0.69$, $P=0.027$). B) Correlation between sTNF-R55 and sTNF-R75 in AA ($r_s=0.70$, $P=0.025$). C) Correlation between sICAM-1 and sE-selectin in AD ($r_s=-0.56$, $P=0.152$). D) Correlation between sICAM-1 and sE-selectin in AA ($r_s=0.78$, $P=0.008$).

positive correlation between sTNF-R55 and sTNF-R75 levels in the AD group ($r_s=0.69$, $P=0.027$) as well as in the AA group ($r_s=0.70$, $P=0.025$), but not in the NAA group or the HC group. The correlation coefficient between the plasma levels of soluble adhesion molecules, sICAM-1, and sE-selectin was not significant in the AD group ($r_s=-0.56$, $P=0.152$) (Fig. 2C), but was significant in the AA group ($r_s=0.78$, $P=0.008$) (Fig. 2D).

Relationship between plasma levels of sICAM-1, sE-selectin, sTNF-R55, or sTNF-R75 and severity of the AD as assessed by the SCORAD

Levels of sICAM-1 were found to be increased in sera from patients with acute exacerbation of atopic eczema (9). Therefore, the relationship between the SCORAD and the plasma levels of sICAM-1, sE-selectin, sTNF-R55, or sTNF-R75 was determined. In AD patients, a significant correlation ($r=0.73$, $P=0.038$) was observed between plasma levels of sE-selectin and the SCORAD (Fig. 3). No correlation could be detected between

sICAM-1, sTNF-R55, or sTNF-R75 and the SCORAD (data not shown).

Discussion

This study shows that sICAM-1 and sE-selectin can be detected in plasma of children whether or not they suffer from atopic disease. Most important, in the case of sE-selectin, but not sICAM-1, a significant correlation was found with an index of disease severity (SCORAD) in the AD group. Inflammatory cytokines, such as TNF- α and interleukin (IL)-4, induce upregulation of several adhesion molecules, including E-selectin and ICAM-1, on endothelial cells located in the target organs of the inflammatory process. After shedding from endothelial cells, these soluble adhesion molecules retain their capacity to bind their receptors, thereby potentially limiting the inflammatory process (2). In our study, no differences could be observed in the plasma levels of these soluble adhesion molecules between members of any of the patient groups tested relative to healthy controls. One possible explanation

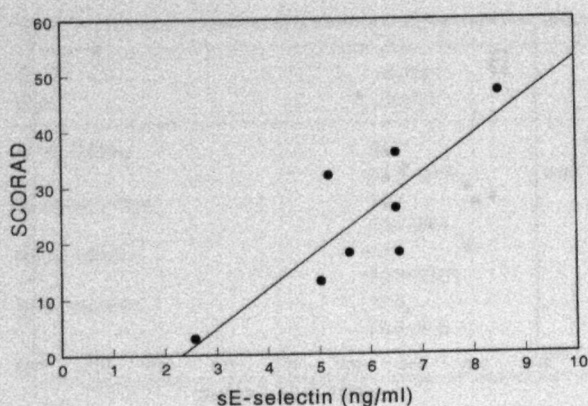


Fig. 3. Correlation between sE-selectin (ng/ml) and SCORAD in AD ($n=8$), with correlation coefficient of $r=0.73$ and $P=0.038$.

is based on the heterogeneity among individuals. Furthermore, we studied patients under relatively stable conditions of their atopic disease, and disease severity may vary within the patient groups.

In the case of adult asthmatic patients, it is disputed whether sICAM-1 and sE-selectin reflect the severity of the inflammation (8) or are related to the allergic status of the patients (5). Our results, showing a correlation between sICAM-1 and sE-selectin in AA, but not NAA, patients, are more compatible with the results of Gosset et al. (5), suggesting that the mechanisms and cells involved in the inflammation in nonallergic asthma are different from those found in allergic asthma. This suggests that adhesion molecules are more important in the inflammation present in allergic asthmatic patients than in nonallergic asthmatic patients.

A second explanation of the lack of differences in the levels of soluble adhesion molecules between healthy controls and the various patient groups might be the relative immaturity of the immune system of the children studied. In several respects, the immune system of neonates and infants has been described as relatively immature as compared to that of adults (34). We were not able to detect age-related differences in the plasma levels of soluble adhesion molecules. Moreover, the age distributions in all patient groups were similar. Therefore, we consider it unlikely that the immaturity of the immune system of the children included in this study caused this lack of difference.

We could not detect TNF- α in plasma of any patient or healthy control studied, probably due to the presumably brief half-life of TNF- α in the circulation (35).

This study considered atopic patients who were generally in stable phases of their disease. In these

patients, no disease-related increases in TNF- α or sICAM-1 were detected. During exacerbation of the disease, increases in the levels of TNF- α and sICAM-1 or sE-selectin were found (8, 9, 11).

Soluble TNF-R in the circulation is considered to be more stable than TNF- α (19). Therefore, it was of interest to study the levels of sTNF-R in these patients as well. We were able to detect sTNF-R55 and sTNF-R75 levels in healthy children similar to those observed in healthy adults (17, 36). For all patient groups, a similar age-related decrease in the levels of sTNF-R was found. For sTNF-R also, no differences could be observed between atopic and nonatopic children. Serum sTNF-R levels were found to be increased only transiently in some diseases, such as systemic lupus erythematosus and rheumatoid arthritis (21, 22). Therefore, levels of sTNF-R in plasma may be increased only during an asthma attack or during exacerbation of atopic dermatitis.

Only in the groups of atopic children was a correlation found between sTNF-R55 and sTNF-R75 levels (both AD and AA groups). In nonatopic children (HC and NAA), all of these molecules could be detected, but no significant correlations were found. A correlation between both sTNF-R levels was also described for other diseases (37–39). Thus, variation in soluble adhesion molecules and sTNF-R levels could reflect disease activity. Further study including patients during exacerbation of the atopic disease is necessary to establish such a relation.

Between plasma sE-selectin levels and the SCORAD in AD patients, a linear relationship was found. The SCORAD is not a marker for deterioration of the disease, but it is a combined symptom score collectively indicating the severity of the disease (28). Such a correlation between sE-selectin levels and the severity of the dermatitis has been found before in adult AD patients by Czech et al. (40). The difference between the correlation coefficients of their study and our study might be due to multiple factors (i.e., different ELISA, children vs adults, different type of severity index). This indicates that sE-selectin is probably associated with disease activity in AD. Further analysis in larger patient groups will permit the determination of the distribution of these sE-selectin levels in relation to severity of disease and possibly support the clinical application of this relation. The lack of consensus on a severity index for asthmatic children did not permit such an analysis in these groups of patients as yet.

In conclusion, in AD patients, a significant correlation was observed between the levels of sE-selectin and the SCORAD. Further study with a larger patient population is necessary to determine

how these molecules change during deterioration of atopic disease. These results will allow a better understanding of the clinical implication and more insight into the inflammatory processes characteristic of these diseases.

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