

Allergens, IgE, mediators, inflammatory mechanisms

A role for eosinophils in the pathogenesis of skin lesions in patients with food-sensitive atopic dermatitis

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Background: Atopic dermatitis is associated with skin and blood eosinophilia, but the role of eosinophils in the pathogenesis of the skin lesions is poorly understood.

Methods: To determine whether eosinophils play a role in the pathogenesis of the skin lesions in atopic dermatitis, we studied the relationship between the severity of the disease and both the number and the extent of activation of eosinophils in 15 patients with food-sensitive atopic dermatitis. Furthermore, this relationship was re-evaluated in eight of these patients who, after a period of elemental diet or total parenteral nutrition, showed significant clinical improvement.

Results: A clear relationship was found between the number of light-density eosinophils and the severity of the disease both during the active disease and after clinical improvement. Furthermore, we describe an adhesion-stimulating activity for eosinophils in patients' plasma, which does not change after recovery.

Conclusions: Taken together, these observations strongly indicate that eosinophils play a pivotal role in the pathogenesis of the skin lesions in atopic dermatitis. In particular, the light-density phenotype seems to be an essential feature of eosinophils involved in this process. The adhesion-promoting activity that we observed in the patients' plasma could be important in the recruitment of eosinophils from the blood into the skin. (*J ALLERGY CLIN IMMUNOL* 1995;96:200-8.)

Key words: Eosinophil, atopic dermatitis, adhesion

Atopic dermatitis (AD) is a distinctive form of eczematous disease occurring during infancy or childhood. It is a chronic condition, which tends to improve, but may recur with a variety of clinical manifestations throughout life.¹

Patients with AD demonstrate an overproduction of IgE, which can be directed against food or inhaled allergens,^{1,2} as well as *Staphylococcus* antigens.³ Although various immunologic defects have been described in patients with AD,^{1,4} the

Abbreviations used

AD:	Atopic dermatitis
APA:	Adhesion-promoting activity
BSA:	Bovine serum albumin
CS:	Clinical score
ECP:	Eosinophil cationic protein
LDEs:	Light-density eosinophils
NDEs:	Normal-density eosinophils
PBS:	Phosphate-buffered saline
PBS-BSA-C:	PBS-BSA-citrate
TPN:	Total parenteral nutrition

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Supported by grants from the MURST (40% and 60%), CNR of Italy (grant 94.02828.CT04) and Associazione Italiana per la Ricerca sul Cancro.

Received for publication July 20, 1993; revised Apr. 1, 1994; accepted for publication Nov. 3, 1994.

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0091-6749/95 \$3.00 + 0 1/1/62057

role played by the effector cells in the pathogenesis of the skin lesions is still poorly understood.

Eosinophilia is common in these patients, and histologic examinations of the skin lesions have revealed early infiltration of eosinophils in addition to an increased number of T cells, mast cells, and basophils.^{1,2,5-7} Furthermore, highly toxic prod-

ucts derived from degranulated eosinophils also have been found both in skin biopsy specimens^{5,7} and in the blood stream.⁸

Taken together, these findings suggest that the eosinophil, a cell endowed with a highly toxic potential, could play a key role in the pathogenesis of skin lesions in AD.

In this study we investigate the relationship between the severity of the disease and the number of eosinophils, the extent of their activation, and the presence in the plasma of factors capable of promoting eosinophil adhesion. Furthermore, we try to relate the changes in these parameters to the clinical improvement that occurred after a period during which the patients were nourished with a strictly eliminatory elemental diet or total parenteral nutrition (TPN).

METHODS

Patients

Fifteen patients with AD (9 boys and 6 girls, ranging in age from 0.5 to 9.5 years) were selected for this study from the population of children affected by AD who were referred to the Institute of the University Pediatric Department of the University of Trieste during the period from February 1991 to April 1992 (15 months).

The diagnosis of AD was made according to the classical criteria of Hanifin.⁹ Although none of our patients underwent placebo-controlled, double-blind food challenge, food allergy seemed reasonably present on the basis of both the anamnestic acute cutaneous reactions to food and the class III or IV positivity of RAST for at least two food antigens among those tested (e.g., lactoglobulin, lactalbumin, casein, ovalbumin, yolk, corn, and soybean components). The severity of the disease was scored in 14 patients at the time of diagnosis and in eight patients after dietetic treatment (see below), on the basis of the degree of erythema, exudation, crusting, and lichenification. For each of these symptoms a score of 0 to 3 was assigned in nine separate sites of the body surface (including head, neck, trunk, legs, arms, and hands). Itching was scored separately as follows: when present only in undressed child (weak intensity itch) = 5; continuous = 10; frenetic with alteration in normal behavior = 15. According to this scoring system, the maximum attributable score was 123 (total clinical score [CS] = 4 items $\times$ 9 sites $\times$ 3 maximum score + 15 maximum itching score = 123). The minimum to maximum range of the score for our patients was 29 to 86. The level of serum IgE was measured (see below) and found to be above normal (for age); the level was 1880 IU/ml on average (minimum to maximum range, 75 to 8280 IU/ml) in the 14 patients tested.

The patients were given either an elemental diet or TPN for an average period of 15 days (minimum to maximum range, 8 to 30 days). The patients were

selected for an elemental diet or TPN because they had severe and diffuse AD that was not responsive to conventional treatment or dependent on oral steroids. Moreover, all of the patients had been previously hospitalized at least once without significant improvement of the disease.

They all gained advantage from the elemental diet or TPN and showed a significant clinical improvement (minimum to maximum score range before treatment, 29 to 86; minimum to maximum score range after treatment 10 to 34). It was possible to analyze the blood of most of the patients before the treatment but of only eight of them after the treatment (aged 0.5 to 6.0 years, 4 boys and 4 girls). The elemental diet (Nutrinaut; Nutricia, Milano, Italy), which was given to five boys and four girls (aged 0.5 to 0.8 years), was made up of (wt/vol): amino acids 13.3%, carbohydrate 68.6%, lipids 15.5%, minerals 2.3%, and vitamins 0.3%. TPN, which was given to four boys and two girls (aged 3 to 6 years), was made up of an amino acid-balanced solution (1.5 to 2 gm/kg/day, Freamine 8.5%; Clinitec, Milano, Italy), fatty acids (20% solution, Intralipid; Pharmacia, Uppsala, Sweden), oligoelements, and vitamins. During the period of the elemental diet or TPN, any antihistamine or steroid-based therapy (topical or systemic) was suspended, whereas a nonspecific topical nonsteroidal treatment was continued for several days.

Controls

Blood samples, drawn (with parents' informed consent) from 10 nonatopic children (age- and sex-matched), were used as controls.

Evaluation of the activated state of the eosinophils

The activation of the eosinophils in AD was tested in two ways: by evaluating the number of light-density eosinophils (LDEs) (eosinophils in their activated form, also known as hypodense eosinophils)¹⁰⁻¹⁸ and by evaluating the plasma level of the eosinophil cationic protein (ECP).

Evaluation of the number of LDEs and normal-density eosinophils

Four to seven milliliters of blood was withdrawn in heparin (Sigma Chemical Co., St Louis, Mo.) 10 to 20 U/ml, with parents' informed consent, at the time of hospital admission. The number of white cells was counted electronically (Coulter Counter ZBI; Coulter Electronics Ltd., Luton, U.K.), and differential counts were carried out on Wright- and Giemsa-stained smears to allow the determination of the absolute number of circulating eosinophils.

The percentage density distribution of the eosinophils was determined by using a Percoll gradient (Pharmacia), according to the method of Cramer et al.,¹⁹ with minor modifications. After the erythrocytes were sedimented by addition of dextran (0.75% final concentration) (Dex-

tran T-500, Pharmacia) in physiologic saline solution, the leukocytes, diluted in phosphate-buffered saline-bovine serum albumin-citrate (PBS-BSA-C), were then centrifuged at 200 *g* for 10 minutes. The cell pellet was suspended at a concentration of 2×10^6 cells/ml in Percoll brought to a density of 1.0853 ± 0.0002 gm/ml as measured at 20° C with a DMA 45 density meter (A. Parr, Graz, Austria) and to an osmolality of 290 mOsm. Four milliliters of this cell suspension was stratified between a 1 ml cushion of 1.1274 gm/ml isotonic Percoll and 1 ml overlay of PBS-BSA-C in 12 × 75 mm polyethylene cylindrical test tubes. Two tubes were prepared for each sample of blood to be analyzed. After a 20-minute centrifugation (1000 *g* calculated from the bottom of the tube) at 18° C in a swinging-bucket rotor, the cell layers on the two interfaces (PBS-BSA-C/Percoll 1.0853 and Percoll 1.0853/Percoll 1.1274) were removed together with the middle phase of Percoll (density = 1.0853). They were collected, washed with PBS-BSA-C, and freed of contaminating erythrocytes by a 30-second hypotonic lysis with 3 ml of 1 mmol/L sodium phosphate buffer, pH 7.4.

Isotonicity was restored by addition of 7 ml of 1.3% NaCl in 1 mmol/L sodium phosphate buffer, pH 7.4. The three cell populations were counted electronically and brought to a cell concentration of 0.2×10^6 /ml in PBS containing 1.2 mmol/L $MgCl_2$, 5 mmol/L KCl, 0.5 mmol/L $CaCl_2$, 5 mmol/L glucose, and 0.1% BSA (PBS+) to prepare cytocentrifuged specimens (Cytospin 2; Shandon, Inc., Pittsburgh, Pa.) and to carry out differential counts after conventional staining. The percentage of LDEs (density at 20° C < 1.0853) was calculated from the absolute number of the eosinophils present in the top interface, taking the total number of eosinophils present in the leukocyte population processed as 100%. The eosinophils present in the other layers (density at 20° C ≥ 1.0853) were assumed to be of normal density. It is noteworthy that only a few cells, mainly eosinophils, were always present in the middle layer. The determination of LDE number was carried out in all patients.

Assays for the plasma levels of ECP and IgE

Plasma level of ECP was measured in 14 patients by a radioimmunoassay (ECP RIA, Pharmacia) according to the method of Venge et al.,²⁰ as recommended by the manufacturer. Determination of ECP was carried out on plasma, prepared within 30 minutes from the heparinized blood collection, rather than serum because the former is thought to be indicative of "in vivo" release of this cationic compound.^{21,22} The radioactivity was counted in a Beckman LS 6000TA liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The serum levels of IgE were measured by ELISA (Phadezim PRIST, Pharmacia). The assay was shown to be reproducible both in triplicate determinations of any sample and in assays carried out on the same sample on different days.

Isolation of NDEs from adult blood

The method described by Cramer et al.¹⁹ was followed. As judged by differential counts, the resulting cell population usually contained 85% to 100% eosinophils (> 98% trypan blue-negative); the remaining cells were neutrophils with dense pyknotic nuclei.

Evaluation of adhesion-promoting activity in the patients' plasma

The presence of an adhesion-promoting activity (APA) in patients' plasma was evaluated as follows: 50 μ l aliquots of a suspension of NDEs, obtained from adult normal blood and brought to a cell concentration of 10^6 /ml, were deposited in replicate wells of a tissue culture plate (Costar, Cambridge, Mass.) and prewarmed for 5 minutes at 37° C. After the addition of 20 μ l of 17.5% to 70% PBS+ diluted plasma (final dilution 2% to 20%) to each well, the plate was placed in a humidifier and incubated for 20 minutes at 37° C. At the end of incubation, the nonadherent cells were removed by gently washing the wells twice with warmed PBS+. The number of adherent cells was determined by estimating their peroxidase content with the 3,3',5,5'-tetramethylbenzidine assay.^{23,24} The reaction produced a soluble, stable end product, which was read spectrophotometrically at 405 nm in a microplate reader (Multiscan MCC/340; Gruppo Flow S.p.A, Milano, Italy). The number of adherent eosinophils, corresponding to a specific peroxidase content, was calculated from a calibration curve, obtained by estimating the total peroxidase activity (extracted from the cells with the cationic detergent cetyl-trimethyl-ammonium bromide) of 2000 to 20000 cells derived from the same batch used in the adhesion assay. APA was expressed as the percentage of adherent eosinophils, with the number of cells originally put into the well taken as 100%. APA was evaluated in 11 of the 15 patients considered.

Statistical analysis

The Graph-pad Instat program (Graph-pad Software, Inc., San Diego, Calif.) was used to calculate *p* values from a one- or two-tailed Student's *t* test on unpaired data and to calculate the linear regression and the correlation coefficient (*r*). Considering the distribution of the points, the Graph-pad Inplot 4 program was used to calculate the correlation coefficient for nonlinear regression (exponential saturation curve and rectangular hyperbola).

RESULTS

Our patients showed the clinical signs and symptoms of pure AD: none had respiratory complications. Nevertheless, the severity of the disease varied widely, as indicated by the CSs reported in Table I. All of the patients had an increased level of IgE (for their age) (minimum to maximum range, 75 to 8280 IU), and peripheral eosinophilia was found in the majority (Table I); patients 14

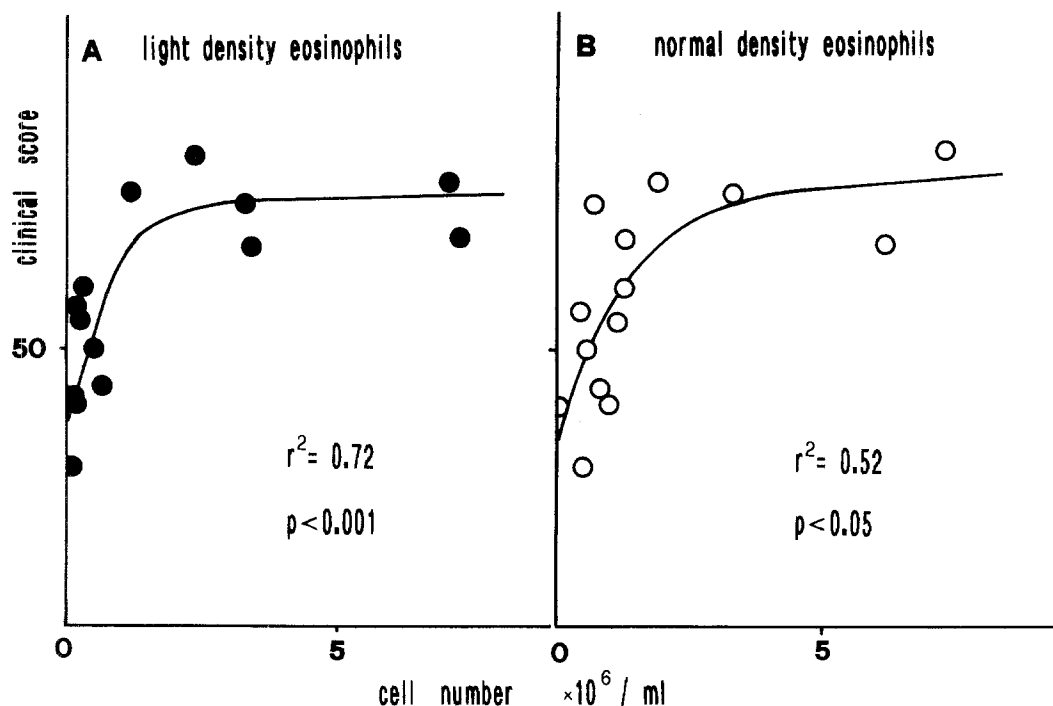


FIG. 1. Relationship between the number of eosinophils per milliliter of blood and the CS. Statistical analysis was performed with the Graph-pad Inplot 4 program. A nonlinear regression analysis for an exponential saturation curve was carried out. Each value represents a single patient.

TABLE I. Characterization of patients affected by AD during active disease by functional and immunologic criteria

	A Patients*	B Normal subjects*	p Value (A vs B)
CS	59.5 ± 4.7 (14)		
Min-max	29 – 86		
Eosinophils (cells × 10 ⁶ /ml)	3.9 ± 0.8 (15)	0.34 ± 0.09 (10)	<0.05
Min-max	0.2 – 9.6	0.04 – 0.68	
ECP (nmol/L)	0.98 ± 0.22 (14)	0.29 ± 0.04 (6)	<0.05
Min-max	0.3 – 3.7	0.15 – 0.43	
APA (%)	21.6 ± 4.0 (11)	9.0 ± 1.9 (8)	<0.05
Min-max	9.0 – 52	3.8 – 18.9	

Min-max, Minimum to maximum range.

*Values are expressed as means ± SEM. Number of patients tested is indicated in parentheses. The *p* values were calculated from one-tailed Student's *t* test for unpaired data.

and 12 showed borderline eosinophilia and a normal eosinophil count, respectively.

The concentration of ECP was significantly increased in plasma of patients with AD (Table I) and was not dependent on the number of circulating eosinophils (data not shown); there is no significant correlation between the ECP level and the severity of the disease (data not shown).

Conversely, Fig. 1, A, shows that the number of

LDEs, calculated for 1 ml of blood, was significantly related to the severity of the disease. When the number of circulating LDEs was higher than 10⁶ cells/ml, the CS leveled off in an exponential saturation curve.

The number of circulating NDEs was related to the CS in a similar way, but the plots were more scattered and the statistical significance was lower (Fig. 1, B). We did not observe any significant

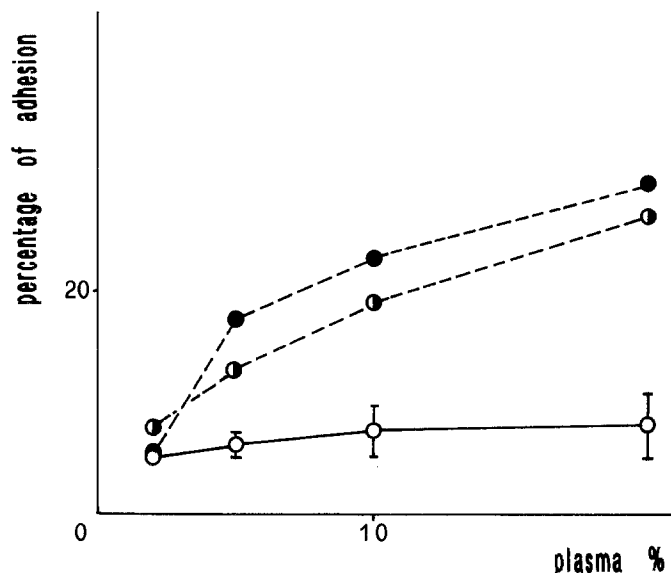


FIG. 2. Dose-response curve of APA (see text) in plasma from two patients and matched control subjects. Four plasma samples were analyzed for control curve (*open circles*), and APA is reported as the mean $\pm$ SEM of determinations carried out in triplicate.

correlation between the CS and the level of IgE, which was, however, high in the plasma of all of the patients (data not shown).

When the adhesion of normal adult eosinophils, with a normal density, was tested on a plastic surface in the presence of 20% plasma obtained from 11 of our patients, an APA was clearly demonstrated. In fact, Table I shows that the patients' plasma increased the adhesion of normal eosinophils more than normal plasma. As shown in Fig. 2, APA was dependent on the plasma dilution in the assay system. It was already detectable when the plasma was 20-fold diluted, whereas the maximal difference with respect to control plasma was obtained at a fivefold dilution (20%). Similar results were obtained when an umbilical vein endothelial cell monolayer was used as substrate (data not shown). APA did not relate significantly to CS (linear regression analysis: APA vs CS, $r = -0.49$), but it was shown to relate inversely to the number of LDEs. Fig. 3, *A*, shows that in the patients tested, the lower the APA value, the higher was the number of circulating LDEs. The number of normodense eosinophils did not show a significant relationship with APA (Fig. 3, *B*).

Table II shows that after a period of elemental diet or TPN (see Methods section), all eight patients experienced a significant clinical improvement as judged by the decrease of the CS values and the concomitant drastic reduction of circulat-

ing eosinophils. The clinical improvement (expressed as the difference between the CS during the active disease and the CS soon after recovery) did not show a statistically significant relationship with the variation of both the APA and the ECP levels (linear regression analysis: ECP vs clinical improvement, $r = 0.1$; APA vs clinical improvement, $r = -0.01$). Actually these parameters did not significantly decrease after the diet, with respect to the values observed during the active phase of the disease; rather, a small increase was observed on average (Table II). After the density of the blood eosinophils was analyzed, we found that the clinical improvement seemed to be strongly related to the extent of the decrease in the number of LDEs (Fig. 4, *A*), but not to that of the number of NDEs (Fig. 4, *B*).

Fig. 4, *A*, shows that the extent of the decrease in the LDE number is related to the variation of the CS. However, the number of LDEs remaining in circulation after the elemental diet or TPN did not frequently fall within the normal range (data not shown) nor was it related to the CS at the recovery stage (linear regression analysis: CS at the recovery stage vs number of LDEs at the recovery stage, $r = 0.4$, $p = 0.33$). However, the percentage decrease in the LDE number (taking the number of these cells during the active stage of the disease as 100%) was significantly related to the clinical improvement (linear regression, $r = 0.72$, $p < 0.05$).

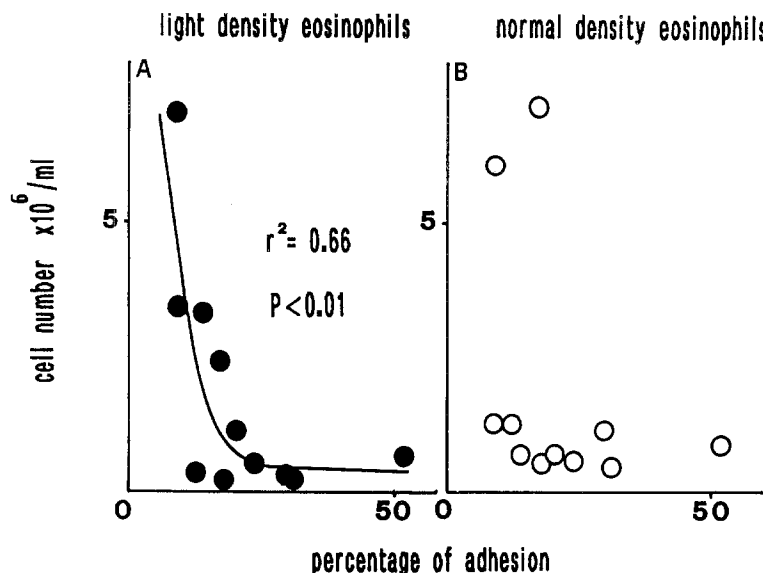


FIG. 3. Relationship between the percentage of APA induced by the patients' plasma on NDEs isolated from normal subjects and the corresponding number of eosinophils per milliliter of patients' blood (A, light density; B, normal density). A nonlinear regression analysis for a rectangular hyperbola was carried out. Each value represents a single patient.

TABLE II. Characterization of patients affected by AD during the active disease and after recovery

	A Active disease	B After recovery	p Value (A vs B)
CS	63.2 ± 6.2 (8)	14.9 ± 4.1 (8)	<0.0001
Min-max	29 – 86	10 – 34	
Eosinophils cells × 10 ⁶ /ml	4.8 ± 1.3 (8)	1.9 ± 0.6 (8)	<0.05
Min-max	0.5 – 8.8	0.4 – 4.5	
ECP (nmol/L)	0.6 ± 0.1 (8)	1.5 ± 0.8 (8)	0.13 (NS)
Min-max	0.2 – 1.0	0.3 – 5.6	
APA (%)	21.7 ± 3.6 (8)	28.2 ± 5.0 (8)	0.32 (NS)
Min-max	12.0 – 25.8	16.9 – 42.9	

Values are expressed as means ± SEM. Number of patients tested is indicated in parentheses. The *p* values were calculated from two-tailed Student's *t* test for paired data. *Min-max*, Minimum to maximum range; *NS*, not significant.

DISCUSSION

In this study we show that in AD a relationship exists between the number of circulating LDEs and the severity of the disease. We observed that in the patients tested, the number of LDEs was proportionally related to the CS up to a value of 10⁶/ml. The relationship between the number of NDEs and the CS, although statistically weaker, probably reflects the association between the overproduction of eosinophils and their activation to the light density state, which, as stated before, is significantly related to the severity of the disease.

These observations suggest, although they do not prove, a functional relationship between the

activity of LDEs and the progression of the skin lesions.

It has been shown that the eosinophils of the allergic donors are primed by cytokines or other factors, to adhere to^{25, 26} and migrate through^{26, 27} an endothelial monolayer. These findings suggest that factors capable of stimulating the adhesion and margination of the eosinophils to the vascular wall could also be present in the plasma of patients with allergy.

As expected, we found an APA in the plasma of our patients, suggesting that the potential exists for the eosinophils to marginate to the vascular wall at a higher level in patients with AD than in normal

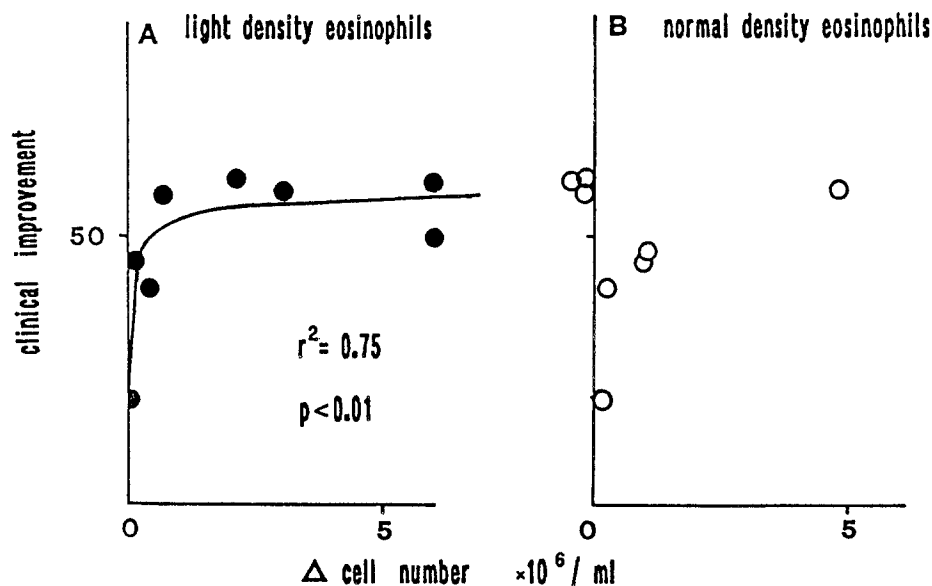


FIG. 4. Relationship between the clinical improvement (calculated as value of the CS during the active disease minus value of the CS soon after the clinical amelioration) and the decrease of circulating eosinophils as referred to 1 ml of blood (A, light density; B, normal density). A nonlinear regression analysis for an exponential saturation curve was carried out. Each value represents a single patient.

subjects. When marginated, eosinophils could better perceive a transendothelial efflux of chemotactic factors and initiate the tissue infiltration. APA was significantly and negatively related to the number of circulating LDEs, which can accord with the existence, in atopic patients, of plasma components capable of increasing the flux of LDEs from the circulation into the tissues and therefore of modulating the number of both blood and tissue LDEs. We speculate that this mechanism could depend on the overproduction of cytokines observed in allergic patients^{28, 29} and even in patients with AD,^{30, 31} or could be identified with the serum chemotactic activities previously described in patients with asthma³² or birch pollen allergy.³³ It would be of interest to characterize, in patients with AD, both the adhesion-promoting plasma factors and the expression of adhesion molecules on the eosinophil surface.

The association between the severity of the clinical conditions and both the number and function of LDEs was further confirmed in eight of our patients who were treated with TPN or an elemental diet. These controlled diets resulted in a significant clinical improvement in our patients, as expected from the data already reported by others^{34, 35} and from our personal experience.³⁶

In these patients the clinical improvement was

related to the decrease in the LDEs in the circulation according to an exponential saturation curve. The number of residual circulating LDEs was found not to be related at all to the CS after recovery, whereas the relative percentage change in LDEs was significantly related to clinical improvement. This suggests that it is the extent of the downregulation of the activation process, leading to the light-density state, that is important for the clinical improvement and not the absolute number of LDEs remaining in the circulation during the recovery stage.

The fact that the amount of plasma ECP did not relate to the severity of disease deserves some comment. We think that a longer period of observation may be necessary to show a decrease in ECP than that required to demonstrate the decrease in circulating LDEs. A different clearance mechanism, in different clinical conditions, for the granule proteins secreted from eosinophils could be, in principle, the reason that some authors succeeded in relating the plasma level of these proteins to the clinical conditions,³⁷⁻³⁹ whereas others failed to do so.⁴⁰ The presence of high ECP plasma levels after clinical improvement, however, cannot exclude the direct involvement of this protein in the progression of the skin lesions because ECP could be continuously released from LDEs into the circula-

tion where it may well be inactivated by plasma components.

In conclusion, the data reported in this study strongly suggest that a relationship exists between the activation of the eosinophils and the severity of the skin lesions in AD. The harmful effect and the direct involvement of eosinophils in the pathogenetic processes of other diseases have been also inferred, on similar bases, by other authors.^{37, 41-43} We speculate that the antigenic burden stimulates some specific clones of T cells to produce interleukin-2³¹ and interleukin-5³⁹ and possibly other cytokines,²⁹ which in turn lead to an increase in both the production and activation of eosinophils.⁴⁴ These cells, primed for adhesion²⁵ and probably undergoing those changes that eventually lead them to the light-density phenotype, marginate in high numbers, particularly at the endothelium of the skin vessels, and in that site perceive the chemotactic stimuli coming from the tissue. These cells, armed with IgE receptors (type II), which they express in high numbers,¹¹ start to infiltrate the target tissue, and in the presence of the specific antigen, secrete their toxic products, which can accumulate and progressively damage the target tissue.

All these observations point to the eosinophil as a cell with a pivotal role in the pathogenesis of AD skin lesions but cannot exclude that a substantial contribution to this process could be made by other cells, such as monocytes and neutrophils, which are known to be primed in AD for the production of superoxide⁴⁵ and leukotrienes,⁴⁶ respectively.

We thank Dr. Bifoni (Blood Bank of the Hospital of Udine) and Dr. Franchi (Blood Bank of the Hospital of Monfalcone) for supplying normal blood and Dr. Bertocin for his technical assistance. We also thank the staff of the Fondazione Carlo e Dirce Callerio and Mr. Harold Wilson Knowles for their help in the revision of the manuscript.

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