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Modulation of CD3-dependent Lymphocyte Proliferation by Extracellular Matrix Proteins in Atopic Dermatitis

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Patients with atopic dermatitis were found to have an about 7-fold increased spontaneous proliferative response of peripheral blood lymphocytes and an about 4-fold elevation of CD3-dependent lymphocyte transformation as compared to normal controls. The CD3-dependent lymphocyte response in patients with severe atopic dermatitis lesions was increased to a lower degree than in those with mild skin lesions. Despite a highly increased CD3-dependent lymphocyte response, the extracellular matrix proteins could induce further co-stimulation of lymphocytes in patients with atopic dermatitis, similar to that in normal controls. However, co-activation by type IV collagen was markedly increased in patients with severe lesions, whereas co-stimulations by both type I collagen and fibronectin were decreased in patients with mild lesions. This finding reflects presumably the changes in lymphocyte subpopulations and their activities related to the recirculation of these cells through the active skin lesions and to the contact of T cells with extracellular matrix proteins. The percentage of CD26-positive lymphocytes was also significantly ($p < 0.05$) increased in patients with severe atopic dermatitis.

These data indicate that helper T cells are excessively activated in atopic dermatitis and that the function of beta-1-integrin receptors underlying the extracellular matrix protein-mediated co-activation of CD3-dependent lymphocyte responses is modified by disease severity. ***Key words: T lymphocytes; collagen; fibronectin.***

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The predominant phenotype of T lymphocytes forming skin infiltrate in atopic dermatitis (AD) lesions has been identified as Th2 cells (1–3), in contrast to Th1 phenotype of CD4+ nickel specific T-cell clones in patients with contact allergy to nickel (4).

These cells in the AD lesions did not only show a lymphokine secretion profile of Th2 cells, i.e. increased interleukin 4 (IL-4) and IL-5 production (5) but also included allergen-specific clones (5, 6) and induced in vitro IgE synthesis by B cells from normal individuals (7). In addition, peripheral blood T cells from atopic patients proliferated in vitro in the presence of one or more allergens, which coincided with elevated titer of serum IgE with specificity towards these allergens (3, 6). Some of the abnormalities in peripheral blood T cell responsiveness in AD patients (8–10) may be due to the cell activation and relative subpopulation shift during lymphocyte recirculation through skin lesions.

The contact of activated CD4+ T cells with connective tissue antigens may alter the responsiveness of peripheral blood lymphocytes (PBL) and their surface markers, including adhesion molecule repertoire as reported previously for psoriasis, another chronic inflammatory skin disease with T cell infiltrates (11).

The purpose of the present paper was to study the in vitro CD3-dependent proliferative response of PBL and its modification by extracellular matrix (ECM) proteins in patients with AD. We also wanted to correlate alterations in these co-stimulatory responses to disease severity, and occurrence of personal and familial atopy.

MATERIAL AND METHODS

Selection of patients

Twenty patients suffering from AD (mean age 31 years, range 17–58 years, 11 males and 9 females), and 20 age- and sex-matched healthy volunteers were studied. The patients were divided into 2 subgroups of severe and mild disease by clinical criteria of disease activity described elsewhere (12). Shortly, six clinical features, i.e. erythema, purulence, excoriations or crusting, dryness or scaling, cracking or fissuring, and lichenification, were graded on a scale of 0 (none) to 3 (severe) for 6 defined body sites: head and neck, anterior and posterior trunk, mid-upper to mid-lower arm (both sides), both hands, mid-thigh and mid-calf (both sides), and both feet. The maximal score was 108; severe cases had a score above 40, whereas mild cases had a score below 36. The extent of the skin lesions was assessed according to the "rule of nines" (12).

It was measured in each defined site by estimating whether none, a third, twothirds, or all of the area was affected. For instance, with the head and neck, investigators calculated the area affected to give a score 0, 3, 6, and 9%; whereas for the upper extremities – 0, 6, 12, and 18%, etc. The extent of disease in mild cases did not exceed 20% of body surface. Other subgroupings of the patients were based on the coexistence of other atopic diseases and by history of familial atopy.

Proliferative assay

Peripheral blood mononuclear cells (MNC) were isolated using Ficoll-Isopaque gradient centrifugation. The MNC preparations were depleted of monocytes by incubation on plastic plates, and the remaining cells were washed in phosphate-buffered saline (PBS). The cells were suspended in complete culture medium: RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin and 10% fetal calf serum. Flat-bottom microtiter plates (Nunc-Schoeller Pharma, Vienna, Austria) were incubated with 1 μg/ml of OKT3 monoclonal antibody (Ortho Pharmaceuticals, Raritan, New Jersey) in PBS at 4°C overnight. Unbound antibodies were washed out with PBS, and next three ECM proteins, namely type I collagen (COL I), type IV collagen (COL IV), and fibronectin (FN), dissolved in PBS to the dose of 10 μg/ml, were incubated in appropriate separate wells at 22°C for 3 h. The plates were washed 3 times in PBS. The suspension of monocyte-depleted MNC, of an average concentration of 10^5 cells per well, was cultured in the coated microtiter plates at 37°C for 4 days. For the last 16 h of the assay, all cultures were pulsed with 1 μCi of [3H]-thymidine (3H-Td) per well. The results are expressed either as arithmetic mean cpm of triplicate cultures, or as relative co-stimulatory index, which is the relative ratio of the ECM protein-induced co-stimulation of CD3-mediated response to CD3-dependent response alone.

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Table I. *Spontaneous and CD3-dependent lymphocyte response (cpm × 10^{-3}, mean ± SEM) in patients with atopic dermatitis and normal controls*

	No. of cases	Spontaneous initial [3H]-thymidine uptake	CD3-mediated T cell [3H]-thymidine uptake
Atopic Dermatitis	20	2.91±0.48**	38.5± 7.0****
Severe	11	2.34±0.53***	25.1± 6.8*
Mild	9	3.61±1.14****	54.9±12.1***
Psoriasis	21	0.77±0.10	8.6± 1.6
Normal	12	0.43±0.10	9.6± 2.9

Differences from normal controls: *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$

Fluorescein activated cell sorter (FACS) analysis

Flow cytometry on MNC isolated as above was performed using fluorescein isothiocyanate conjugated monoclonal antibodies to human CD26, CD29 (beta-1 chain), CD49b (very late antigen-2, VLA2, alpha-2 chain) and CD49c (VLA-3, alpha-3 chain) (prod. Immunotech S.A., Marseille, France). The results were expressed as the mean percentage of labelled lymphocytes and as the mean channel of fluorescence.

Statistics

Significance of the difference in mean values obtained in all experimental groups was calculated using Student's *t*-test. Results given in the text represent a mean ± SEM.

RESULTS

Spontaneous 3H-Td incorporation by PBL was markedly increased in patients with AD, both with severe and mild lesions as compared to healthy controls (Table I). No significant increase was found in patients with psoriasis.

The CD3-dependent lymphocyte response in AD patients was higher than in normal subjects and patients with psoriasis (Table I). CD3-dependent lymphocyte proliferation in patients with severe AD was markedly elevated as compared to normal controls; however, it was much lower ($p < 0.05$) than in patients with mild lesions.

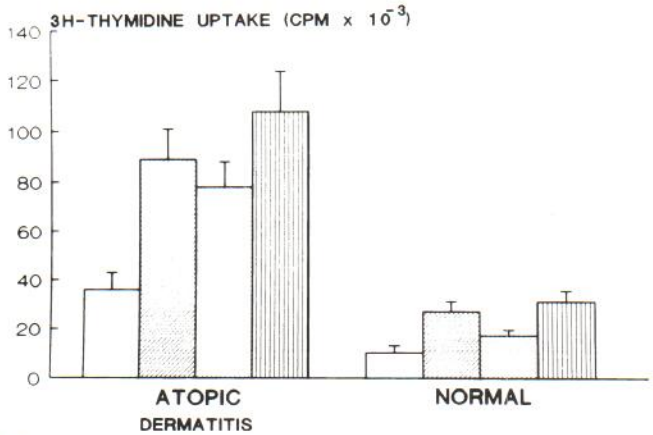

Fig. 1. Absolute 3H-thymidine incorporation (mean ± SEM) by lymphocyte cultures exposed to OKT3 antibodies alone and during co-activation of the CD3-dependent response by extracellular matrix proteins in patients with atopic dermatitis and healthy subjects.
Statistical difference between atopic dermatitis and normals (*t*-test); □ anti-CD3 alone, $p < 0.01$; ▨ type I collagen, $p < 0.005$; ▩ type IV collagen, $p < 0.001$; ▥ fibronectin, $p < 0.02$.

The ECM proteins alone did not stimulate the lymphocyte proliferation in any group, since response to ECM proteins requires previous activation of the CD3 molecule – T cell receptor complex of T cells. 3H-Td incorporation in AD patients in the presence of COL I (2750 ± 450 cpm), COL IV (2880 ± 500 cpm), and FN (3120 ± 470 cpm) was similar to spontaneous lymphocyte response in the absence of the ECM proteins (2910 ± 480 cpm). Respective values of 3H-Td-incorporation in normal controls (COL I, 510 ± 110 cpm; COL IV, 470 ± 80 cpm; FN, 460 ± 120 cpm) were not different from non-stimulated lymphocyte cultures (430 ± 100 cpm). The ECM proteins induced co-activation of the CD3-dependent lymphocyte response in both AD patients and normal controls, despite the fact that this CD3-dependent lymphocyte response was already increased several-fold (Fig. 1).

The relative index of co-stimulation by COL I was slightly lower than in AD patients as compared to that in healthy subjects. This co-stimulation index by COL IV was somewhat higher in AD than in the control group, whereas that induced by FN was similar in both groups (Table II). None of these relative indices of co-stimulation by the ECM proteins in AD patients was statistically significantly different as compared to the normal population. In patients with severe AD lesions the lymphocyte co-stimulatory responses to COL IV and FN were increased as compared to the remaining patients with mild AD (COL IV, 4.03 ± 0.57 versus 1.56 ± 0.29, $p < 0.005$; and FN, 5.89 ± 0.91 versus 2.49 ± 0.43, $p < 0.05$).

The relative co-stimulation index in the presence of COL I (49 ± 12% of normal) and FN (58 ± 10% of normal) in patients with mild AD lesions was significantly lower ($p < 0.05$) than in normal individuals.

No statistical differences in the values mentioned above were found between patients with coexisting bronchial asthma (or other atopic symptoms) (anti-CD3 alone, 35.9 ± 6.6 × 10^3 cpm; and COL I, 62 ± 7%; COL IV, 136 ± 46%; FN, 106 ± 31% compared to normals) and patients with AD only (anti-CD3 alone, 43.0 ± 13.7 × 10^3 cpm; and COL I, 89 ± 29%; COL IV, 134 ± 43%, FN, 95 ± 25% compared to normals) or between cases with (anti-CD3 alone, 40.5 ± 12.5 × 10^3 cpm; and COL I, 86 ± 27%; COL IV, 164 ± 54%; FN, 127 ± 32% compared to normals) and without (anti-CD3 alone, 38.1+6.9 × 10^3 cpm; and COL I, 60 ± 6%; COL IV, 98 ± 7%; FN, 71 ± 6% compared to normals) familial history of atopy. Patients with familial atopy had a more severe course of the disease.

Since the marked ECM-mediated co-stimulatory response of CD3-dependent lymphocyte proliferation was found in AD patients, spontaneous adherence of lymphocytes to FN- and COL IV-coated plates as well as phytohaemagglutinin (PHA)-stimulated adherence were determined; these were within normal ranges (data not shown). In addition, FACS analysis has shown the normal percentage of cells expressing integrin beta-1 chain (CD29) and alpha-2 chain (VLA2 = collagen receptor) and alpha-3 chain (VLA3 = fibronectin, collagen and laminin recep-

Table II. *Relative co-stimulation index (cpm of anti-CD3 and extracellular matrix protein co-stimulated culture / cpm of anti-CD3 stimulated culture) in patients with severe and mild atopic dermatitis and healthy subjects (mean ± SEM)*
C.I. = co-stimulation index.

		Extracellular matrix protein		
		Col I	Col IV	FN
Atopic Dermatitis (total, 20)	C.I.	2.70±0.57	2.84±0.68	4.20±0.82
	% of normal C.I.	74±15	134±32	98±19
Severe (11)	C.I.	3.47±0.66	4.03±0.57**	5.89±0.91
	% of normal C.I.	95±18	191±27	138±24
Mild (9)	C.I.	1.76±0.43*	1.56±0.29	2.49±0.43*
	% of normal C.I.	49±12	74±14	58±10
Normal Controls (12)	C.I.	3.65±0.61	2.12±0.31	4.27±0.59

Statistical significance of the difference (*t*-test): *COL I and FN, mild lesions versus normal controls ($p < 0.05$) and FN; **COL IV, severe lesions versus normal controls ($p < 0.02$).

tor) (Table III). The percentage of CD26-positive cells, i.e. these expressing surface antigen presumably responsible for the adherence of lymphocytes to COL IV, was slightly ($p<0.05$) elevated in patients with AD as compared to healthy controls. The increased percentage of these cells was found almost exclusively in patients with severe AD lesions. The distribution of CD26 antigen was of similar density in both groups (Table III).

DISCUSSION

Two recent findings demonstrating that CD4+ T cells cloned from AD dermis are allergen-specific (7) and that these T cell clones have the cytokine production profile of Th2 cells (5), allow for a better understanding of the role of T cells in the pathomechanism of AD.

These cells do not only promote specific-IgE production but can recognize inhalatory and food allergens in the dermis, become activated and mediate cytotoxic reactions in response to these antigens (3, 6, 13).

Table III. *Surface integrin markers (mean ± SEM) on peripheral blood lymphocytes in patients with atopic dermatitis and healthy subjects*
MCF = mean channel of fluorescence.

		Surface marker			
		CD26	CD29	CD49b	CD49c
Atopic Dermatitis (total, 20)	% cells	56± 2.4*	74±2.7	2±0.4	10±1.6
	MCF	89± 6.7	65±6.2	56±4.9	41±4.2
Severe (11)	% cells	65± 2.4***	71±3.9	1±0.4	7±2.1
	MCF	95±10.2	62±8.1	63±7.8	41±5.1
Mild (9)	% cells	45± 2.0	77±3.7	3±0.7	13±2.3
	MCF	82± 8.7	69±9.3	50±8.3	42±7.0
Normal controls (20)	% cells	45± 2.9	75±3.1	3±0.7	14±2.7
	MCF	116±14.1	70±6.9	57±8.3	49±6.9

Statistical significance of the difference (*t*-test): *AD versus normal controls ($p < 0.05$); ***severe lesions versus normal controls ($p < 0.001$)

In our study, an excessive spontaneous lymphocyte proliferative response of PBL (7-fold increase) as well as a highly increased CD3-dependent lymphocyte response (4-fold) were found. It is likely that these T cells have been activated in vivo presumably by allergens (atopens), as reported previously (6), or that the overall suppressive effect of T cells on the CD3-induced transformation is diminished in AD (14). This is also in agreement with the concept of the prevalence of T helper function in patients with AD (5, 7, 12). T cells showing Th2 helper activity may expand with time in the peripheral blood during their recirculation, which alters overall lymphocyte activation through CD3 receptor.

This enhanced lymphocyte proliferation was also demonstrated as co-activation with the ECM proteins, since the presence of the ECM proteins induced further stimulation of the CD3-dependent lymphocyte response, i.e. both COL I and COL IV induced about 3-fold enhancement, FN about 4-fold enhancement in the total AD group. Since the ECM proteins react with adequate VLA molecules on the lymphocyte membrane serving as receptors for these proteins, this increased co-stimulation may indicate that the function of these receptors belonging to the beta-1 integrin family is up-regulated in patients with AD. Thus, in addition to the CD3-stimulus responding T cells the second signal triggered by all ECM proteins has been enhanced.

Some alterations of co-activation by the ECM proteins of the CD3-dependent lymphocyte response occurred in patients with severe and mild AD lesions. The relative ratio of the co-stimulatory lymphocyte response to COL I and FN in patients with mild disease was reduced by about 50% as compared to the co-stimulation index of healthy subjects, although CD3-dependent response of these cells was highly increased. There is no good explanation for this phenomenon. The decreased proportion in the peripheral blood of T cells responding to COL I and FN because of their arrest in the lesional skin when symptoms of disease are mild or down-regulation of T cell receptors for COL I and FN are not fully satisfying interpretations.

In contrast, co-stimulation by COL IV of CD3-mediated response was significantly increased in patients with severe AD

lesions, whereas co-stimulations by COL I and FN were similar to the relative index of normal controls. Excessive co-stimulation by COL IV might be caused by in vivo contact of lymphocytes with COL IV in the basement membrane zone of the epidermis, when these cells recirculate through lesional AD skin. This interpretation might be supported by some tendency towards the increased percentage of CD26-positive T cells in patients with severe AD lesions as compared to healthy subjects. The discrepancy between lymphocyte responsiveness to COL I and COL IV is evident, and it is similar to that observed in other chronic inflammatory skin diseases involving T cells, such as psoriasis (11, 15–18).

Migration of T cells from the capillary vessels to the skin lesions seems to alter the T cell responsiveness and, possibly, repertoire of T cell markers and adhesion molecules. In consequence, in skin inflammatory diseases, some T cell subsets in the peripheral blood may expand following repeated recirculation through the diseased skin.

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