

Chronologic analysis of in situ cytokine expression in mite allergen-induced dermatitis in atopic subjects

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Patch testing with crude dust mite extracts, after removal of horny epidermal layers, was performed on normal-appearing skin of nine adult patients with atopic dermatitis who had high mite-specific IgE antibody levels. Positive skin reactions were observed in seven subjects. Skin biopsy specimens were obtained from positive reaction sites at 2, 6, 12, 24, and 48 hours after allergen challenge and subjected to histologic and immunohistochemical studies and extraction of RNA. Perivascular infiltration of small mononuclear cells began at 2 hours, followed by eosinophilic infiltration at 6 hours, which peaked at 24 and 48 hours. Increased expression of IL-4 messenger RNA was detected with reverse-transcription polymerase chain reaction at 12 and 24 hours, whereas immunohistochemical staining with anti-IL-4 antibody showed positive reactions in connective tissue around infiltrating cells after 2 hours. Expression of IL-5 and tumor necrosis factor- α mRNA was upregulated 2 hours after an application of allergen. Interferon- γ mRNA was not detected. These findings suggest the crucial role of T_H2 -type cytokines in initiating eosinophil infiltration of mite allergen-induced dermatitis in patients with atopic dermatitis. (J ALLERGY CLIN IMMUNOL 1995;96:1069-75.)

Key words: Atopic dermatitis, mite allergen, patch test, cytokines, RT-PCR

Atopic dermatitis (AD) skin lesions are characterized by the infiltration of CD4⁺ T cells and antigen-presenting cells, such as Langerhans' cells and macrophages.¹ Although eosinophils are not prevalent histologically, immunohistochemical studies can reveal extensive dermal deposition of eosinophil granule-derived proteins, including the major basic proteins.² Hamid et al.³ recently demonstrated that acute and chronic AD skin lesions were associated with increased activation of IL-4 and IL-5 genes and that initiation of acute skin inflammation was predominantly associated with increased IL-4 expression, whereas maintenance of chronic inflammation was associated with a predominance of IL-5 expression and eosinophil infiltration. However, biopsy findings of such already established skin lesions do not always provide satisfactory information about the dynamic inter-

Abbreviations used

AD:	Atopic dermatitis
RT-PCR:	reverse transcription polymerase chain reaction
TNF- α :	Tumor necrosis factor- α

action of participating cells in initiating eosinophil infiltration in AD skin lesions.

Although patch testing with mite allergen on atopic subjects with high serum IgE against mite allergen is known to induce acute eczematous lesions, which are characterized by extensive eosinophil infiltration,⁴⁻⁶ few studies have examined, chronologically, the development of eosinophil infiltration and cytokine expression in such induced dermatitides. Therefore we analyzed mite allergen-induced dermatitis chronologically.

METHODS

Patients

Nine adult inpatients with clinically severe AD and high IgE RAST scores against mite allergens gave informed consent to undergo patch testing and biopsies (Table I). The patient group consisted of seven men and

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TABLE I. Profile of study patients with AD

Patient No.	Age (yr)	Sex	Serum IgE (mg/dl)	RAST score		Patch test					
				DF	DP	0 hr	2 hr	6 hr	12 hr	24 hr	48 hr
1	26	M	629	6	6	—	+	+	ND	+	++
2	21	M	18,300	6	6	—	—	—	ND	±	—
3	20	M	8,520	6	6	—	—	—	ND	—	—
4	21	F	2,810	6	6	—	+	+	ND	++	++
5	26	M	7,760	6	6	—	+	+	+	+	++
6	28	M	14,400	6	6	—	—	++	ND	+	++
7	23	F	1,280	5	5	—	++	+	++	++	ND
8	20	M	4,870	6	6	—	—	++	+	++	ND
9	20	M	25,100	5	5	—	—	—	—	++	ND

DF, *D. farinae*; DP, *D. pteronyssinus*; ND, not done.

two women, aged 20 to 28 years (mean age, 22.8 ± 3.1 years). Their serum IgE levels varied from 629 to 25,100 IU/ml (mean level, 9297 ± 8373 U/ml), and the scores of their IgE RAST against mite antigens (*Dermatophagoides pteronyssinus* and *D. farinae*) were either 5 (50 to 99.9 UA/ml) or 6 (more than 100 UA/ml). The control group consisted of six age-matched nonatopic healthy volunteers.

Patch testing

Crude mite extract was a kind gift from Drs. Mori and Okudaira (Department of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo) and was prepared according to the methods of Miyamoto et al.⁷ White petrolatum was used as the vehicle, and final concentration of crude mite extract was adjusted to 1%. Patch testing was performed with Finn chambers on normal-appearing skin. The horny layer of skin in the tested sites was removed by repeated application and stripping of adhesive tape. The vehicle alone served as a control. The reactions after 0, 2, 6, 12, 24, and 48 hours were evaluated according to the criteria of the International Contact Dermatitis Research Group.

Processing of biopsy tissues

Biopsy specimens were obtained with a disposable a punch-biopsy apparatus from the positive patch test sites. Half of each specimen was subjected to extraction of RNA; a quarter was fixed according to the AMeX method⁸ for histochemical staining and in situ hybridization; and another quarter was frozen, stored at -80°C , and subjected to immunohistochemical staining.

Immunohistologic staining

The 5 mm frozen sections were incubated with monoclonal mouse anti-human IL-4 and IL-5 antibodies (Genzyme, Cambridge, Mass.) (1:50 dilution in phosphate-buffered saline) after incubation with levamisole and were visualized with a Vecstatin ABC-Alkaline

Phosphatase Kit and Vector Red (Vector Laboratories, Burlingame, Calif.).

Reverse-transcription polymerase chain reaction (RT-PCR)

In this study only biopsy tissues from patients 4 and 8 were subjected to RT-PCR study. RNA was extracted from freshly biopsied tissues with the QuickPrep micro mRNA purification Kit (Pharmacia, Uppsala, Sweden). Complementary DNA was synthesized with SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, Md.) and random primers. Oligonucleotides for cDNAs of G3PDH, IL-4, and IL-5 were purchased from Clontech Laboratories (Palo Alto, Calif.). Another pair of oligonucleotides for cDNA of IL-4, IL-5, and tumor-necrosis factor- α (TNF- α) was designed according to the method of Okamoto et al.⁹ and synthesized with the methoxy-phosphoramidite method on an Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.) (Table II). Complementary DNA was suspended in 15 ml of 50 mmol/L Tris/HCl (pH 7.3), 1.5 mmol/L MgCl₂, 200 nmol/L each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 500 pmol/L each primer, and 0.2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). PCR conditions were set as follows: 95°C for 60 seconds for denaturing, then at 58°C for 60 seconds for annealing, and at 72°C for 60 seconds for primer extension. This program was repeated 30 times in a programmable DNA Thermal Cycler (Perkin-Elmer Cetus). Further incubation for 5 minutes at 72°C was performed to complete the extension after the last cycle. Then, 1 ml of the product was taken and subjected to the second PCR, the protocol of which was the same as the first PCR, except that the total volume was 10 ml and the primer concentration was 50 pmol/L. Five-milliliter aliquots of the amplified products were electrophoresed in 5% polyacrylamide gel. In the event that the products were saturated after 30 cycles, fewer cycles (e.g., 10 or 20) were attempted.

TABLE II. A panel of primers for PCR

Primer pair	Sequence	Location
IL-4 (1)	5': ATGGGTCTCACCTCCCAACTGCT 3': CGAACACTTTGAATATTTCTCTCAT	456 bp
IL-4 (2)	5': CACAAGTGCATATCACCTT 3': GCTCGAACACTTTGAATATT	386 bp
IL-5	5': ATGAGGATGCTTCTGCATTG 3': CTCATTGGCTATCAGCAG	193 bp
TNF- α	5': CAGAGGGAAGAGTTCCTCCAG 3': CCTTGGTCTGGTAGGAGACG	325 bp
G3PDH	5': TGAAGGTCGGAGTCAACGGATTTGGT 3': CATGTGGGCCATGAGGTCCACCAC	983 bp

RESULTS

As shown in Table I, seven of nine patients with AD had positive reactions to the mite allergen, and the vehicle alone did not elicit positive reactions. None of six nonatopic subjects had positive reactions.

Representative histologic findings are shown in Fig. 1. Inflammatory cells were scarce before the application of mite allergen (Fig. 1, A). Two hours after allergen challenge, perivascular infiltration of several small mononuclear cells and some eosinophils was observed, and these cells were attaching to the endothelial cells of small vessels in the upper dermis (Fig. 1, D). Perivascular eosinophil infiltration was noted at 6 hours and became more prominent from 24 to 48 hours with occasional small mononuclear cells (Fig. 1, E and F). Epidermal spongiotic changes became noticeable at 6 hours (Fig. 1, B), and a small epidermal eosinophilic vesicle was observed at 48 hours (Fig. 1, C).

RT-PCR analysis showed increased expression of IL-4 after 24 and 48 hours in tissue from patient 4 (Fig. 2). The expected 456 bp band in polyacrylamide gel was visible only after the second amplification. Therefore several pairs of primers for amplification of a known concentration of IL-4 cDNA were compared, and the primer pair reported by Okamoto et al.,⁹ IL-4 (2) (Table II), was found to be the most suitable (data not shown). With this primer pair, increased expression of IL-4 was again revealed after 24 hours in patient 8, although the doubling of PCR was still necessary. The expression of G3PDH was shown to be stable during the time course. In patient 8 expression of IL-5 was noted after 2 hours and increased after 12 and 24 hours after 10 cycles of the second PCR. TNF- α increased in a time-dependent manner after 6 hours after 10 cycles of the second PCR. Expression of interferon- γ mRNA was not detected at all (data not shown).

Immunohistochemical staining with anti-IL-4 antibody revealed spotty presence of IL-4 along the vascular area and occasional infiltrating cells at 2 hours after the application of mite allergen (Fig. 3). IL-4⁺ cells at this time point seemed to be mast cells, as judged by their structure. The staining intensities with anti-IL-4 antibody increased, time-dependently, but it is noteworthy that the majority of infiltrating cells remained unstained at 24 hours after allergen challenge. Histologically, the positive cells are supposed to be mast cells and T cells, and unstained cells are supposed to be mainly eosinophils. Staining with anti-IL-5 antibody revealed approximately the same results (data not shown).

DISCUSSION

It has been demonstrated that characteristic eczematous reactions, resembling acute AD lesions clinically, can be induced by patch testing with mite allergens in patients with AD.⁴⁻⁶ Patch testing with mite allergen induces percutaneous entry of mite allergen and seems to be a more suitable method for studying eczematous reactions in patients with AD than other skin tests, including prick tests and intradermal skin tests. Tanaka et al.⁵ reported that patch testing of mite allergen produced positive reactions in 14 of 20 patients with AD (70%) and in 13 of 14 patients with AD (92.9%) and high RAST titer against *D. farinae*. Bruynzeel-Koomen et al.⁶ reported that patch testing of mite allergen produced positive reactions in 12 of 15 patients with AD (80%), whereas all of the nonatopic subjects showed negative reactions. Our patch testing results confirmed those previous reports. These data indicate the essential role of IgE antibody specific to mite allergens in the pathogenesis of mite-induced dermatitis.

The involvement of eosinophils in formation of acute lesions of AD is controversial. Wakita et al.¹⁰

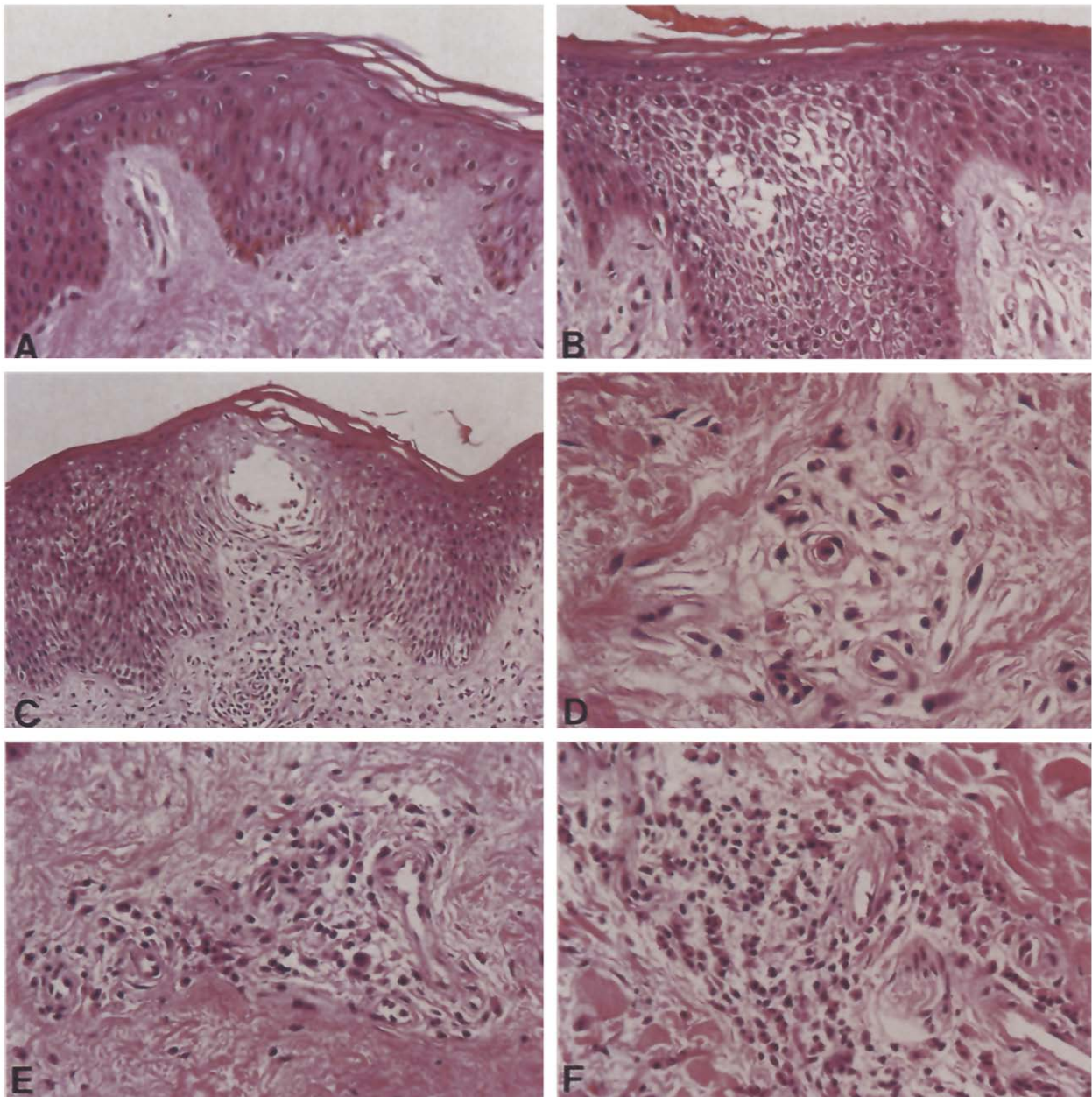


FIG. 1. Representative histologic findings of mite allergen-induced dermatitis. **A**, Control. **B**, Six hours after patch test. Note epidermal spongiotic changes. **C**, Forty-eight hours. Epidermal vesicle consisting of eosinophils. **D**, Two hours. Several perivascular small mononuclear cells. Note the presence of eosinophils attached to the vascular endothelium. **E**, Six hours. Perivascular eosinophil infiltration along with occasional small mononuclear cells. **F**, Forty-eight hours. Note the prominent eosinophil infiltration.

reported small, but significantly greater, numbers of eosinophils in acute lesions of AD than in chronic lesions, which were occasionally infiltrated with eosinophils. On the other hand, Hamid et al.³ reported greater numbers of eosinophils in chronic lesions of AD than in acute lesions. In our study the histologic findings at the patch test sites re-

vealed massive eosinophil infiltration, which is much more prominent than in previous reports on both acute and chronic AD lesions. Bruynzeel-Koomen et al.⁶ suggested that eosinophils might play an important role in developing eczematous reactions to mite allergens. When compared with the previous reports of late-phase reactions,¹¹ the

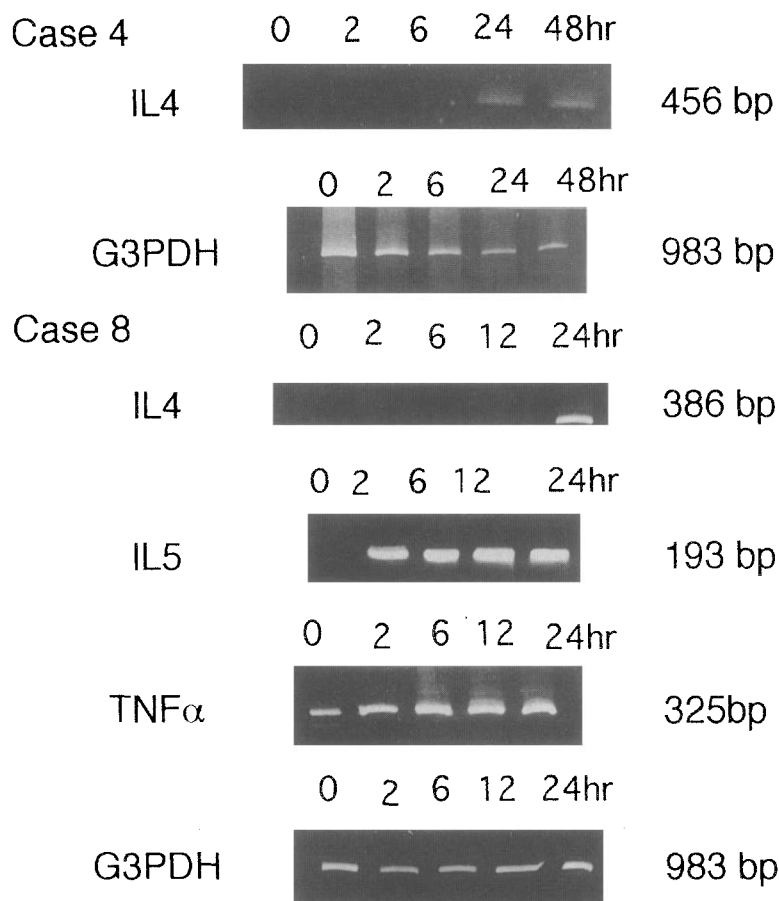


FIG. 2. Detection of cytokine mRNA by RT-PCR. As for IL-4, IL-5, and TNF- α , the products after two cycles of PCR are shown. The band corresponding to IL-4 is visible at 12 and 24 hours after application of mite antigen. Expression of IL-5 and TNF- α is increased during the time course.

histologic findings of patch tests showed induced reactions differ in the following two aspects. First, infiltration of neutrophils is not observed. Second, spongiotic changes in the epidermis are exclusively seen in patch test-induced lesions. Our sequential histologic analysis revealed that 2 hours after antigen challenge, infiltration of small mononuclear cells and some eosinophils occurred, and these cells attached to the endothelial cells in the upper dermis. Perivascular eosinophil infiltration then starts at 6 hours and becomes more prominent at 24 to 48 hours. Epidermal spongiotic changes are noticeable at 6 hours. These histologic changes differ from those of acute AD lesions in that eosinophil infiltration is predominant and T-cell infiltration is rather sparse. Therefore chronologic analysis of the patch testing sites is considered to be useful for investigating the initiation of eosinophil infiltration after percutaneous entry of mite allergen.

The dominant expression of T_{H2}-type cytokines,

such as IL-4 and IL-5, is thought to play an essential role in the pathogenesis of AD. In fact, several reports have shown the presence of T-helper lymphocytes with T_{H2} characteristics in AD skin lesions.¹²⁻¹⁴ Upregulation of gene expression of T_{H2}-type cytokines IL-4 and IL-5 in mite allergen-induced patch test lesions, as shown by RT-PCR, support these data and may indicate that such cytokines are important in initiating eosinophil infiltration. Hamid et al.³ recently demonstrated that acute and chronic AD skin lesions were associated with increased activation of IL-4 and IL-5 genes.

Interestingly, chronic AD skin lesions were associated with a predominance of IL-5 expression and increased eosinophil infiltration. Although cells expressing mRNA of IL-4 and IL-5 are not defined in this study, the earlier expression of IL-5 might indicate that this cytokine is also crucial for initiating eosinophil infiltration. Hamid et al.³ detected cells expressing mRNA for IL-4, even in

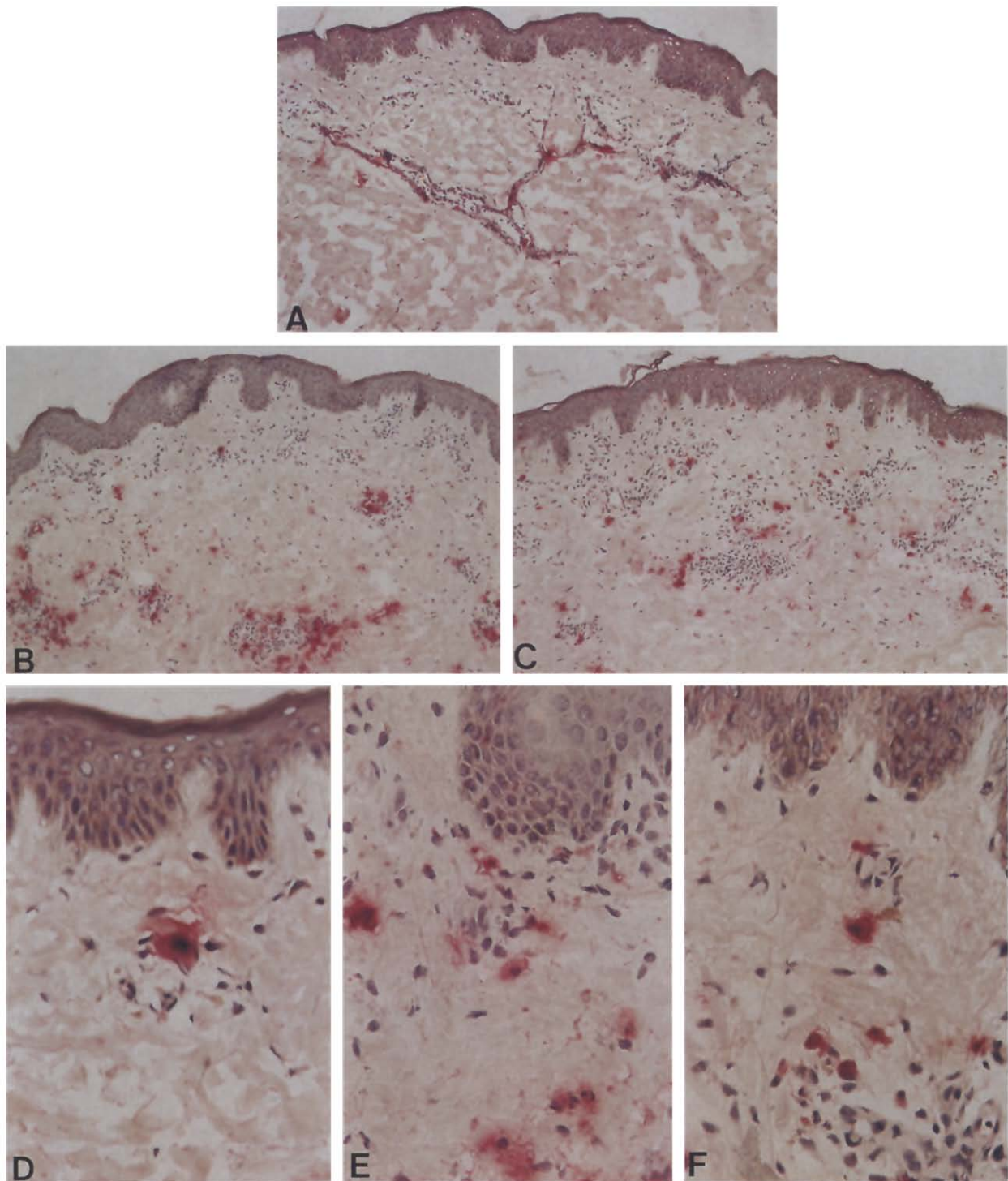


FIG. 3. Staining patterns with anti-IL-4 antibody. **A**, Two hours after the patch test. **B**, Six hours. **C**, Twenty-four hours. **D**, Two hours. Positive cell is presumably mast cell. **E**, Six hours. **F**, Twenty-four hours. Majority of infiltrating cells remain unstained.

uninvolved AD skin. The lack of IL-4 gene expression by RT-PCR in AD control skin in our study might be due to either low levels of mRNA expression for IL-4 or sampling bias.

It has been recently demonstrated by immunohistochemical studies that in acute or subacute AD skin lesions, mast cells are one major source of IL-4.¹⁵ In our immunohistochemical study IL-4 was

detected as early as 2 hours after allergen challenge, before the gene expression. Therefore IL-4 at this time point might be already formed and stored inside mast cells and released by binding of mite allergen to cell surface IgE antibodies. This finding is important when considering the currently well established theory that IL-4 plays a critical role in selecting T_{H2} cells that predominate in an allergic response.¹⁶ Increased expression of TNF- α mRNA was detected after 6 hours. It remains unknown whether stored or de novo synthesized TNF- α acts dominantly in formation of acute AD skin lesions, because immunohistologic study with anti-TNF- α antibody has not been done. There is a possibility that increased expression of these cytokines merely reflects the positive feedback regulation after their release. But the effects of FK506¹⁷ and cyclosporine¹⁸ that inhibit the transcription of cytokines on acute lesions of AD suggest the importance of de novo synthesis of the cytokines. Interferon- γ mRNA favoring the development of T_{H2} cells^{13, 19} was not detected at all. Aside from T_{H2} cells, mast cells and eosinophils have been shown to have a capacity to release IL-4 and IL-5 and to express mRNA.²⁰⁻²² Nevertheless, it is necessary to identify cells expressing mRNA of IL-4 and IL-5 in mite allergen-induced dermatitis, and in situ hybridization with riboprobes is now in progress.

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