

Changes in eosinophil and leukocyte infiltration and expression of IL-6 and IL-7 messenger RNA in mite allergen patch test reactions in atopic dermatitis

Nobuo Yamada, MD,^a Motoshi Wakugawa, MD,^a
Shoji Kuwata, MD, PhD,^b Hidemi Nakagawa, MD, PhD,^a
and Kunihiko Tamaki, MD, PhD^a Tokyo, Japan

Patch testing with crude dust mite extracts after removal of horny layers was performed on normal-appearing skin of 11 adult patients with atopic dermatitis and high mite-specific IgE antibody scores. Positive skin reactions were observed in 9 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 studies and extraction of messenger RNA (mRNA). Perivascular infiltration of small mononuclear cells began at 2 hours and was followed by eosinophilic infiltration at 6 hours, and the number of eosinophils continued to increase at 24 and 48 hours. In addition to the increased expression of IL-4, IL-5, and tumor necrosis factor- α mRNA during the time course detected by reverse-transcription polymerase chain reaction, mRNA of IL-6 and IL-7 was also up-regulated. After the removal of test patches with mite allergen, the number of eosinophils started to decrease in a time-dependent manner. Histopathologic findings at 48 hours after removal showed lymphocyte-dominant infiltration intermingled with occasional eosinophils. These mite allergen patch test reactions may provide a useful model for studying the pathogenesis of atopic eczema, especially with regard to the initiation of eosinophil infiltration and the alternative increase in lymphocytes. (J Allergy Clin Immunol 1996;98:S201-6.)

Key words: Atopic dermatitis, eosinophils, mite allergen, patch test, IL-6, IL-7, reverse-transcription polymerase chain reaction

Grewe et al.¹ studied cytokine patterns expressed in eczematous lesions and found that 85% of skin samples from chronic lesions contained increased levels of interferon- γ (IFN- γ) messenger RNA (mRNA) whereas increased IL-4 mRNA expression was observed only in 25% to 30%. They proposed a two-phase model for the pathogenesis of atopic dermatitis (AD), an early phase and a second phase. In the early phase, T_{H2} cytokines are crucial for the initiation of atopic eczema. In the second phase, T_{H1}-like cytokines such as IFN- γ predominate. They then studied the chronological analysis of cytokine expression patterns in mite-

Abbreviations used

AD:	Atopic dermatitis
cDNA:	Complementary DNA
CME:	Crude mite extract
IFN- γ :	Interferon- γ
RT-PCR:	Reverse transcription polymerase chain reaction

allergen-induced dermatitis.² They showed an increase in IL-4 and IL-2 transcripts but not IFN- γ in 24-hour patch test reactions and an increase in IFN- γ and IL-2 and a decrease in IL-4 transcripts in 48-hour reactions, suggesting that a switch from T_{H2}-like to T_{H1}-like cytokine response occurs between 24 and 48 hours after application of mite allergen. They proposed that the switch of T_{H2} to T_{H1} is due to IL-12, because increased expression of the p35 subunit of IL-12 mRNA was observed in 24-hour reactions.

From the ^aDepartment of Dermatology and ^bDepartment of Immunohematology, Faculty of Medicine, University of Tokyo.

Reprint requests: Hidemi Nakagawa, MD, PhD, Department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Copyright © 1996 by Mosby-Year Book, Inc.
0091-6749/96 \$5.00 + 0 1/0/76960

TABLE I. Profile of patients with AD

Patient no.	Age (yr)	Sex	Serum IgE (mg/dl)	RAST score		Reaction (hr)							
				DF	DP	0	2	6	12	24	48	72	96
Group A													
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		
Group B													
10	25	M	24,500	6	6	-				+	++	++	++
11	20	M	8,930	6	6	-				++	++	++	++
Time after removal (hr)										0	24	48	72

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

TABLE II. Panel of primers for RT-PCR

Primer pair		Sequence	Length (bp)
IL-6	5'	GAATCCTTCTCCACAAGCG	316
	3'	GAATCCAGATTGGAAGCATCC	
IL-7	5'	AACCAGCTGCAGAGATCACC	209
	3'	GCTACTGGCAACAGAACAAGG	
G3PDH	5'	TGAAGGTCGGAGTCAACGGATTGTT	983
	3'	CATGTGGGCCATGAGGTCCACCAC	

Our previous data³ showed increased expression of IL-4 mRNA after 24 hours, which continued to increase even in 48-hour reactions. IL-5 mRNA also continued to increase in patch test reactions from 24 to 48 hours. Although we could not detect IFN- γ consistently, the switch of T_{H2} to T_{H1} cytokine responses did not seem to occur between 24 and 48 hours in our study. This discrepancy may be attributable to the preparation and concentration of mite allergen, to the method of stripping-off of the stratum corneum, or to the patients examined.

In the current study, we have demonstrated that the dense eosinophilic infiltration in 24-hour patch test reactions gradually disappears after the removal of the mite allergen. This model, with histopathologic observation of the transition of major infiltrating cells, would be important for studying the early and second phases of AD. Furthermore, we investigated the expression of IL-6 and IL-7 mRNA, because IL-6 is reported to enhance production of IgE and exaggerate allergic inflammation such as in asthma, and IL7 might be

associated with the proliferation of T lymphocytes in situ at the site of inflammation in skin.

METHODS

Patients

As shown in Table I, 11 adult in-patients with clinically severe AD with high IgE RAST scores against mite allergens were studied after obtaining informed consent for the following patch tests and biopsies. Patients 1 through 9 of 11 (group A) were the same patients as listed in our previous report.³ The patient group consisted of 9 men and 2 women aged 20 to 28 years. Their serum IgE levels varied from 629 to 25,100 IU/ml, and their scores of IgE RAST against mite antigens (*Dermatophagoides pteronyssinus* and *D. farinae*) were 5 (50 to 99.9 unit allergen/ml) or 6 (>100 UA/ml).

Patch testing

Crude mite extract (CME) was a gift from Dr. Akio Mori and Dr. Hirokazu Okudaira (Department of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo) and was prepared by using the methods of Miyamoto et al.⁴ White petrolatum was used as the

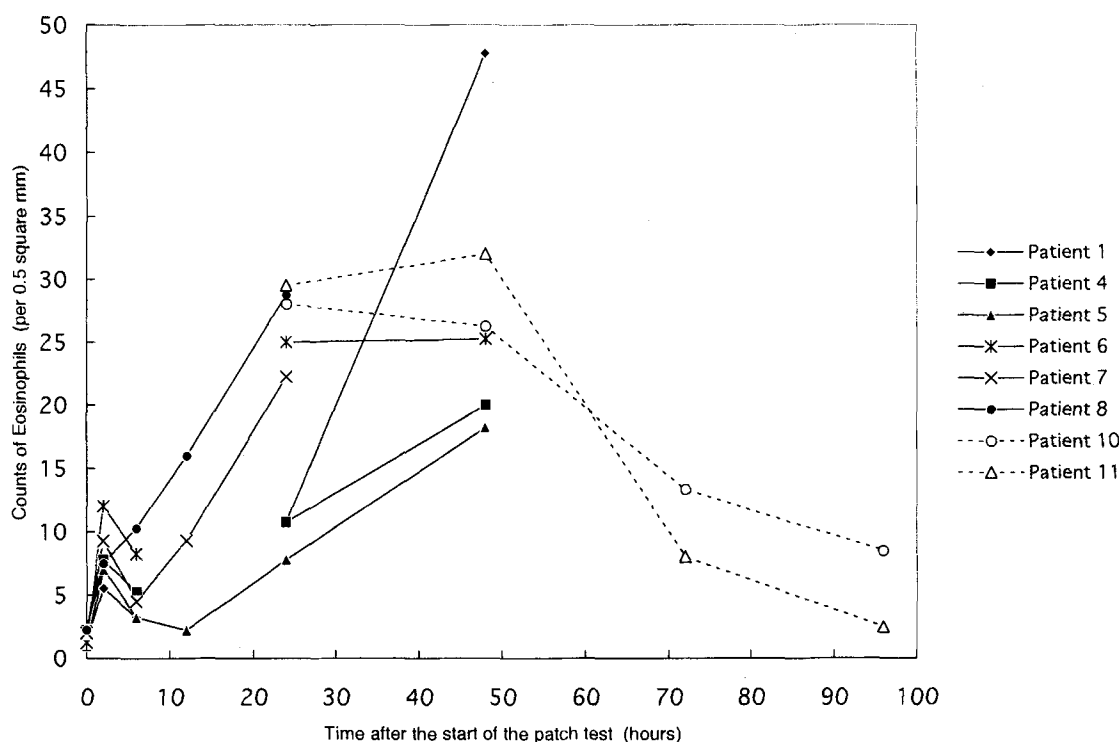


FIG. 1. Eosinophils counts per 0.5 mm² in 4 µm section stained with hematoxylin and eosin.

vehicle, and the final concentration of CME was adjusted to 1% (weight/weight). Patch testing was performed with Finn Chambers® (Epitest Ltd. Oy, Tuusula, Finland) on normal-appearing skin. The horny layer of skin in tested sites was removed by repetitive application and stripping of adhesive tapes. 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. For patients 10 and 11 (group B) the mite allergen was removed after 24 hours, and then after 24, 48, 72, and 96 hours the tested sites were biopsied.

Processing of biopsy tissues

Biopsy specimens were obtained by using a disposable punch-biopsy apparatus from the positive patch test sites. Half of each specimen was subjected to extraction of RNA; a quarter was fixed with the AMeX methods for histochemical staining and in situ hybridization; and another quarter was frozen, stored at -80°C, and subjected to immunohistochemical staining. For counting of the numbers of eosinophils, 4 µm tissue sections were stained with hematoxylin and eosin.

Reverse-transcription polymerase chain reaction (RT-PCR)

In the current study, specimens only from patient 4 were subjected to RT-PCR study. RNA was extracted from freshly biopsied tissues by using a QuickPrep micro

mRNA purification kit (Pharmacia, Uppsala, Sweden). Complementary DNA (cDNA) was synthesized with SuperScript reverse transcriptase and random primers. Oligonucleotides for cDNAs of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were purchased from Clontech Laboratories (Palo Alto, Calif.) Primers for IL-6 and IL-7 were designed with GeneWorks software (IntelliGenetics, Mountain View, Calif.) and synthesized by using the methoxy-phosphoramidite method on an Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.) (Table II). cDNA was suspended in 15 µl of 50 mmol/L TrisHCl (pH 7.3), 1.5 mmol/L MgCl₂, 200 nmol/L of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 500 pmol/L of each primer, and 0.2 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) For detection of IL-6, AmpliWax PCR Gem (Perkin-Elmer Cetus) was used to reduce nonspecific bands according to the protocol for AmpliWax Gem. The RT-PCR condition was set as follows; 95°C for 60 seconds for denaturing, 60°C for 60 seconds for annealing, and 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 µl of the product was taken and subjected to the second RT-PCR, the protocol of which was the same as the first RT-PCR except that the total volume was 10 µl and the primer concentration

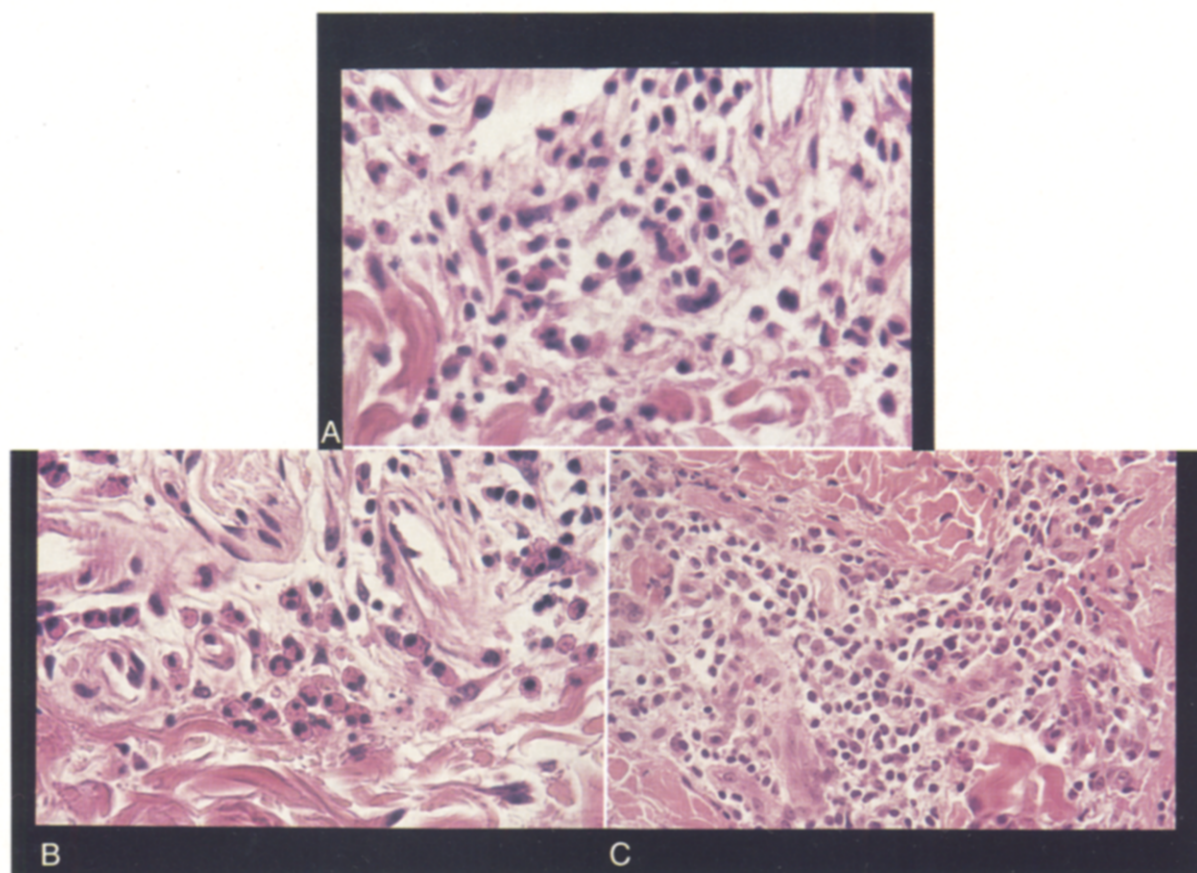


FIG. 2. Representative histologic findings of mite allergen-induced dermatitis. **A**, Twenty-four hours after application of mite allergen. Majority of infiltrating cells are eosinophils. **B**, Forty-eight hours after application of mite allergen. Note dense infiltration of eosinophils. **C**, Forty-eight hours after removal of mite allergen after application for 24 hours. Dense cellular infiltration persists, but majority of cells are small and mononuclear. Apparent eosinophils with granules are sparse.

50 pmol/L. Five-microliter aliquots of the amplified products were electrophoresed in 5% polyacrylamide gel. In case the product, after 30 cycles, was saturated, fewer cycles (such as 10 and 20 cycles) were tried.

RESULTS

Nine of 11 patients with AD showed positive reactions to the mite allergen; the vehicle alone did not elicit positive reactions. Histopathologic changes to 48 hours were described in our previous report. In brief, moderate-degree perivascular infiltration was composed mainly of small mononuclear cells, and occasionally some eosinophils were observed attaching to the endothelial cells of small vessels in the upper dermis 2 hours after allergen challenge. Perivascular eosinophil infiltration was noted at 6 hours and became more prominent from 24 to 48 hours, with occasional small mononuclear cells.

For quantitative analysis, numbers of eosino-

phils per 0.5 mm^2 in a $4 \mu\text{m}$ section were counted under a microscope. Four areas of $0.5 \text{ mm} \times 1 \text{ mm}$ with relatively dense eosinophilic infiltration were chosen on each tissue slide, and the arithmetical mean of the four counts were blotted (Fig. 1). After the removal of mite allergen in patients 10 and 11, the number of eosinophils decreased, and perivascular infiltration composed mainly of small lymphoid cells became prominent. Representative histologic findings are shown in Fig. 2.

RT-PCR analysis showed increased expression of IL-6 after 6, 12, and 24 hours (Fig. 3). The expected 316 bp band in the polyacrylamide gel at 0 and 2 hours was not visible after the second amplification of 30 cycles. Expression of IL-7 was noted after 2 hours and increased after 12 and 24 hours after the second RT-PCR. The expression of G3PDH was detected after the first RT-PCR and was shown to be stable during the time course.

DISCUSSION

Our previous study³ could not demonstrate the switch from T_{H2} to T_{H1} cytokine mRNA expression during the entire time course of patch testing with mite allergen, in contrast to the finding by Grewe et al.,² who showed that this switch occurred between 24 and 48 hours. Apart from the differences of methods and patients used, this discrepancy may be due in part to the inflammation patterns occurring in the patch test reaction sites. In our previous study, even at 48 hours after the patch testing, massive eosinophilic infiltration was the main histologic finding. In the current study we have demonstrated the dense eosinophilic infiltration in 24-hour patch test reactions, which is not usually observed even in biopsy specimens from acute lesions of atopic dermatitis. The histologic findings in patch test reactions 48 or 72 hours after the removal, of predominant lymphocytes and sparse mingled eosinophils, are compatible with those observed in biopsy specimens of acute lesions of AD. Marked deposition of eosinophil-granule-derived proteins, major basic protein and eosinophilic cationic protein, observed by immunohistologic staining (data not shown), is also common in lesions of AD.

Immunohistochemical studies in these lesions suggested that the production of IFN- γ was enhanced as evidenced by the expression of HLA-DR and ICAM-1 in lesional keratinocytes (our unpublished data, 1995). Therefore, clarification of the relation between the histologic changes from the predominance of eosinophils to lymphocytes and mRNA expression of T_{H2} and T_{H1} cytokines is necessary and is now in progress.

Only several kinds of cytokines have been investigated for their expression in situ during the initiation phase of atopic dermatitis. We could not find any previous reports about IL-6 and IL-7.

IL-6 has a wide variety of biologic functions. In association with allergic inflammation, enhancement of production of immunoglobulins, activation of T cells, and proliferation of keratinocytes are considered important. IL-6 and IL-4 play important roles in the production of IgE. IL-4 is an essential factor for class switching in the presence of a second signal through CD40 on B cells from the CD40 ligand on T cells. IL-6 enhances production of IgE by B cells in an autocrine fashion, because B cells secrete IL-6. Production of IL-6 by B cells is stimulated by IL-4. Therefore, IL-4 is associated with the initiation of IgE production, whereas IL-6 is associated with continuous production of IgE.

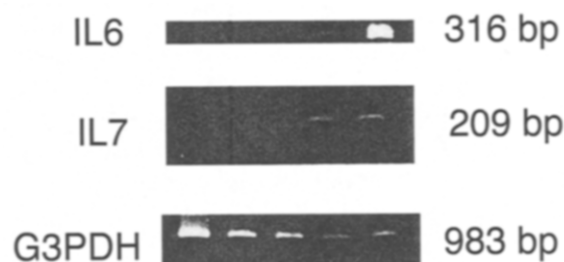


FIG. 3. Detection of cytokine mRNA by RT-PCR. Expression of IL-6 and IL-7 after two RT-PCR cycles is shown.

In patients with asthma, Gosset et al.⁵ reported increased IL-6 production by alveolar macrophages and peripheral blood monocytes. In patients with AD, Toshitani et al.⁶ reported increased IL-6 production by peripheral blood T cells. But production of IL-6 by peripheral blood monocytes derived from patients with AD exhibited no difference compared with those from normal subjects. They suggested that the activated subsets of T cells producing IL-6, rather than monocytes, are important in the pathogenesis of AD.

In the current study, increased expression of IL-6 was detected from 12 to 24 hours in mite allergen patch test reactions. Expression of IL-6 in AD is supposed to explain the prolonged production of IgE with IL-4. We previously reported increased expression of IL-4 in patch test reactions after 24 hours. The proliferation of keratinocyte, which is histologically observed as acanthosis in chronic lesions, may also be associated with IL-6.

Candidates for the source of IL-6 in mite-antigen-induced dermatitis are many. Not only T and B lymphocytes and monocytes but also eosinophils,⁷ mast cells,⁸ and keratinocytes are reported to have the potential to secrete IL-6. We did not identify the cells producing IL-6 in mite allergen-induced dermatitis. Further study with anti-IL-6 antibody or in situ hybridization is necessary.

IL-7 was originally identified as a growth factor for B cell progenitors and subsequently has been shown to exert proliferative effects on T-cell progenitors and mature peripheral T cells.⁹ Overexpression of IL-7 leads to marked lymphoproliferation in skin, as shown by experiments in transgenic mice.¹⁰⁻¹²

Major sources of IL-7 are stromal cells in bone marrow and the thymus. Aiba et al.¹³ reported the presence of dermal cells in mice analogous to thymic nurse cells. The cultured dermal cells secrete IL-7. Human keratinocytes express IL-7 mRNA and release IL-7 protein in biologically relevant amounts.¹⁴ IL-7 gene expression in kera-

tinocytes is up-regulated by IFN- γ and abrogated by ultraviolet B radiation.¹⁵ The keratinocyte-derived IL-7 serves as a growth factor for the neighboring dendritic epidermal T cells in mice.¹⁶

Our results showed trace amounts of IL-7 expression even in normal-appearing skin of patients with AD and increases in IL-7 mRNA within a few hours after application of mite allergen. Neither up-regulation of IL-7 mRNA in AD skin lesions nor association between IL-7 and allergic skin diseases has yet been reported. IL-7 is supposed to exert multiple effects to amplify inflammation of dermatitis in atopic subjects. First, IL-7 works as a growth factor for in vivo primed antigen-specific T cells and enhance their proliferation in skin. IL-7 enhances proliferation of B cell progenitors, eosinophil progenitors¹⁷ in bone marrow, and mast cells. IL-7 up-regulates expression of IL-6¹⁸ and IL-8¹⁹ by human monocytes.

Expressions of mRNAs of other cytokines, chemokines, and signal transducers are now under investigation, with use of the same material obtained from patch test reactions. The chronologic changes of the expressions of such molecules, considered with the histopathologic chronologic changes, would be useful for the clarification of the pathogenesis of AD.

We thank Dr. Akio Mori and Dr. Hirokazu Okudaira at the Department of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo, for a kind gift of crude mite extract, and Masahiro Hayakawa for technical assistance.

REFERENCES

- Grewe M, Gyufko K, Schopf E, Krutmann J. Lesional expression of interferon- γ in atopic eczema. *Lancet* 1994; 343:25-6.
- Grewe M, Walther S, Gyufko K, Czech W, Schopf E, Krutmann J. Analysis of cytokine pattern expressed in situ in inhalant allergen patch test reactions of atopic dermatitis patients. *J Invest Dermatol* 1995;105:407-10.
- Yamada N, Wakugawa M, Kuwata S, Yoshida T, Nakagawa H. Chronologic analysis of in situ cytokine expression in mite allergen-induced dermatitis in atopic subjects. *J Allergy Clin Immunol* 1995;96:1069-75.
- Miyamoto J, Ishii A, Sasa M. Successful method for mass culture of the house dust mite, *Dermatophagoides pteronyssinus* (Troussart, 1897). *Jpn J Exp Med* 1975;45:133-8.
- Gosset P, Tsicopoulos A, Wallaert B, Joseph M, Capron A, Tonnel AB. Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. *Am Rev Respir Dis* 1992;146:768-74.
- Toshitani A, Ansel JC, Chan SC, Li SH, Hanifin JM. Increased interleukin 6 production by T cells derived from patients with atopic dermatitis. *J Invest Dermatol* 1993;100: 299-304.
- Hamid Q, Barkans J, Meng Q, Ying S, Abrams JS, Kay AB, et al. Human eosinophils synthesize and secrete interleukin-6. *Blood* 1992;80:1496-501.
- Bradding P, Feather IH, Wilson S, Barding PG, Heusser CH, Holgate ST, et al. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects: the mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 1993;151:3853-65.
- Chazen GD, Pereira GM, LeGros G, Gillis S, Shevach EM. Interleukin 7 is a T-cell growth factor. *Proc Natl Acad Sci USA* 1989;86:5923-7.
- Uehira M, Matsuda H, Hikita I, Sakata T, Fujiwara H, Nishimoto H. The development of dermatitis infiltrated by gamma delta T cells in IL-7 transgenic mice. *Int Immunol* 1993;5:1619-27.
- Rich BE, Campos-Torres J, Tepper RI, Moreadith RW, Leder P. Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. *J Exp Med* 1993;177:305-16.
- Samaridis J, Casorati G, Traunecker A, Iglesias A, Gutierrez JC, Müller U, et al. Development of lymphocytes in interleukin 7-transgenic mice. *Eur J Immunol* 1991;21:453-60.
- Aiba S, Nakagawa S, Hara M, Tomioka Y, Deguchi M, Tagami H. Cultured murine dermal cells can function like thymic nurse cells. *J Invest Dermatol* 1994;103:162-7.
- Heufler C, Topar G, Grasseger A, Stanzl U, Koch F, Romani N, et al. Interleukin 7 is produced by murine and human keratinocytes. *J Exp Med* 1993;178:1109-14.
- Ariizumi K, Meng Y, Bergstresser PR, Takashima A. IFN-gamma-dependent IL-7 gene regulation in keratinocytes. *J Immunol* 1995;154:6031-9.
- Takashima A, Matsue H, Bergstresser PR, Ariizumi K. Interleukin-7-dependent interaction of dendritic epidermal T cells with keratinocytes. *J Invest Dermatol* 1995; 105(Suppl):50S-53S.
- Vellenga E, Esselink MT, Straaten J, Stulp BK, De Wolf JT, Brons R, et al. The supportive effects of IL-7 on eosinophil progenitors from human bone marrow cells can be blocked by anti-IL-5. *J Immunol* 1992;149:2992-5.
- Alderson MR, Tough TW, Ziegler S, Grabstein KH. Interleukin 7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes. *J Exp Med* 1991; 173:923-30.
- Standiford TJ, Strieter RM, Allen RM, Burdick MD, Kunkel SL. IL-7 up-regulates the expression of IL-8 from resting and stimulated human blood monocytes. *J Immunol* 1992;149:2035-9.