

Skin-derived aeroallergen-specific T-cell clones of Th2 phenotype in patients with atopic dermatitis

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T-cell lines have been derived from aeroallergen-induced eczematous patch test sites of patients with atopic dermatitis (AD). Biopsy specimens were obtained 24 hours after allergen application to the skin, and only in vivo-activated T cells were propagated. From one patient, the T-cell lines were subcloned at 0.3 cells per well. With allergen-induced interleukin (IL) production, all clones tested (n = 13) were found to be specific for the allergen, producing IL-4 and granulocyte-macrophage-colony-stimulating factor. No IL-2 or interferon- γ was found. The allergen-specific proliferative response of these clones, although the response was dependent on exogenous IL-2 or IL-4, also proved that all clones were allergen specific. Under optimal stimulation (aCD3 plus phorbol myristate acetate), 15% of the clones appeared to be of Th0 phenotype and 70% of Th2 phenotype. In 15% of the clones, IL-4 was produced in the absence of IL-2, IL-5, or interferon- γ . Supernatants of all clones tested induced IgE production by B cells from normal non-atopic donors. The T-cell lines of the other patient demonstrated similar results; allergen-specific proliferation was dependent on exogenous IL-2 or IL-4 and stimulation with aCD3 plus phorbol myristate acetate demonstrated that the T cells in these lines were of the Th2 phenotype. In conclusion, our data reveal that, in AD, percutaneous sensitization to aeroallergens may occur and indicate that allergen-specific Th2 type T cells may be responsible for the high levels of (specific) IgE found in 80% of patients with AD. (J ALLERGY CLIN IMMUNOL 1992;90:184-92.)

Key words: Atopic dermatitis, allergen-specific Th2 cells, epicutaneous patch tests, Dermatophagoides pteronyssinus

AD is a chronic, eczematous skin disease, starting in infancy and characterized by remissions and exacerbations. More than 80% of the patients with AD have elevated levels of serum IgE, with specificity to various environmental allergens¹ to which they also demonstrate immediate-type skin reactions.² However, the relationship between allergy and the pathogenesis of the skin lesions in AD is not clear.

Apart from immediate-type skin reactions after intracutaneous application of aeroallergens like GP and house dust mites (for example, Dpt,) patients with AD may also demonstrate delayed-type (positive after 24 to 48 hours), eczematous skin reactions to these allergens after epicutaneous application.³⁻⁷ A positive EPT reaction to aeroallergens is specific for AD, and the histopathology of the skin of patients with both active AD and an EPT reaction reveals an infiltration of activated (HLA-DR⁺) (mainly) CD4⁺ T cells and APCs like CD1⁺ and CD14⁺ cells in the dermis. Other inflammatory cells consist mainly of eosinophils.³ Recently, it has been demonstrated that epidermal LCs from patients with AD and elevated serum IgE levels express an FcR for IgE, which binds IgE in vivo.⁸ In contrast to IgE⁻ LCs, IgE⁺ LCs can present allergen to autologous T cells from peripheral blood.^{9, 10} Furthermore, there was a significant correlation between in vitro allergen presentation by IgE⁺ LCs and the presence of delayed eczematous skin reactions to these

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allergens.¹⁰ This finding suggests that the EPT to aeroallergens is mediated by IgE⁺ LCs and T cells.

Therefore, the above-mentioned EPT reaction to aeroallergens was used as a model to study the role of allergens in the pathogenesis of AD. T cells were cloned from punch biopsy specimens obtained 24 hours after elicitation of an EPT with *Dpt*.

The aim of this study was to characterize the phenotype, specificity, and function of these skin-derived T-cell clones in relation to the pathogenesis of AD.

MATERIAL AND METHODS

Reagents

Two types of CM were used. Both types consisted of RPMI 1640 supplemented with NaHCO₃ (2 mg/ml) (Seromed; Biochrom KG, Berlin, Germany), supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2 mmol/L) (GIBCO, Grand Island, N.Y.). One type contained 10% of pooled human serum (blood group AB: rhesus positive) (ABS). The other type contained 10% FCS (GIBCO). rIL-2 was a kind gift from Dr. E. Liehl (Sandoz, Vienna, Austria); rIL-4 was a kind gift from Dr. J. E. de Vries (DNAX, Palo Alto, Calif.); rIL-2 and rIL-4 are both used in concentrations of 50 U/ml unless it is otherwise stated. GaM IgG was obtained from Cappel (Organon Teknika Corp., West Chester, U.K.), Leu-4 (aCD3) from Becton Dickinson (Mountain View, Calif.), and FITC-conjugated aCD4, a-gamma/delta TCR, a-alpha/beta TCR, as well as phycoerythrin-conjugated aCD8, from Immunotech (Marseille, France). GaH-IgE-biot and AP-strept were obtained from TAGO (Burlingame, Calif.). Monoclonal a-IgE antibodies (I27)¹¹ were a kind gift of Dr. J. Pène (Unicet, Dardilly, France). The IgE standard (CFTK 92103) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Freeze-dried *Dpt* and GP (mixture II) were a kind gift from Haarlems Allergenen Laboratorium (Haarlem, The Netherlands). Tissue-culture plates were obtained from Costar (Cambridge, Mass.), and culture flasks, tubes, and pipettes were obtained from Falcon (Becton/Dickinson, Lincoln Park, N.J.).

Patients

Two adult patients (CF4 and CF6) with AD (diagnosis in accordance with the criteria of Hanifin and Rajka¹²) were used for this study after giving their informed consent. (Table I) The EPT for *Dpt* (10,000 AU/ml) was considered positive when erythema and papules were present.

T cell cloning procedure

Clinically, noninvolved skin (from the upper part of the back) was patch tested with allergen.³ After 24 hours, the patch test site was cleaned and sterilized, after which punch biopsy specimens were obtained (with 1% xylocaine as a local anesthetic). The biopsy specimens were minced with two scalpels; no proteolytic enzymes were used. Skin fractions from each biopsy specimen were divided over four wells in a 96-well flat-bottom plate containing (1) only CM

Abbreviations used

AD:	Atopic dermatitis
GP:	Grass pollen
<i>Dpt</i> :	<i>Dermatophagoides pteronyssinus</i>
EPT:	Epicutaneous patch test
APC:	Antigen-presenting cells
LC:	Langerhans cells
CM:	Culture media
GaM:	Goat antimouse
TCR:	T cell receptor
a-alpha/beta TCR:	Anti-alpha/beta TCR
GaH-IgE-biot:	Biotinylated goat antihuman IgE
RT:	Room temperature
LST:	Lymphocyte stimulation test
AP-strept:	Alkaline-phosphatase-conjugated streptavidin
FcR:	Fc receptor
ABS:	Human AB rhesus serum
FCS:	Fetal calf serum
a-IL:	Anti-interleukin
rIL:	Recombinant IL
FITC:	Fluorescein isothiocyanate
EBV-B:	Epstein-Barr virus transformed B cells
PBS:	Phosphate-buffered saline
PMA:	Phorbol myristate acetate
INF-γ:	Interferon-gamma
GM-CSF:	Granulocyte-macrophage-colony-stimulating factor
a-IgE:	Anti-IgE

ABS, (2) CM ABS supplemented with antigen (*Dpt*, 50 µg/ml), (3) CM ABS supplemented with rIL-2 and rIL-4, and (4) CM ABS supplemented with antigen, rIL-2, and rIL-4; rIL-4 was added to avoid selection of IL-2 dependent T cells. After 21 days, a clear outgrowth of T cells could be found in culture system No. 3 above (lines CFTS4:2 and CFTS6:2) and system No. 4 above (lines CFTS4:3 and CFTS6:3). Subsequently, the cells in the responding wells were counted in an 0.1% trypan blue solution. From patient CF4, viable cells were subcloned in 96-well U-bottom plates under limiting dilution conditions with allogenic, irradiated (4000 rad) EBV-B cells (10⁴ per well)¹³ in CM supplemented with rIL-2 and rIL-4. No mitogens were used. Cells from responding wells were restimulated with immobilized aCD3 antibodies.¹⁴ Therefore, 96-well flat-bottom plates were coated with GaM IgG (20 µg/ml in 0.05 mol/L of Tris buffer solution, pH 9.6) for 1 hour at RT. Wells were washed twice with PBS and subsequently incubated with aCD3 (50 ng/ml in PBS supplemented with 2% FCS, pH 7.4) for 1 hour at RT. Subsequently, the aCD3 solution was aspirated, and cells were added and incubated with CM containing rIL-2 and rIL-4. Clones of about 10⁵ cells were transferred into 24-well plates, expanded (with

rIL-2 and rIL-4), and tested for antigen specificity in an LST. More than 50 clones of each cell line could be stored in liquid nitrogen.

Fluorescence-activated cell sorter analysis

Clones were screened for CD4 or CD8 and TCR expression. Heretofore, 10^5 cells were incubated in a 96-well U-bottom plate for 30 minutes on ice with FITC-conjugated aCD4, and phycoerythrin-conjugated aCD8, FITC-conjugated α - α /beta TCR, or FITC-conjugated α -gamma/delta TCR. Subsequently, the cells were washed three times (250 g, for 3 minutes at 4°C), fixed in 2% paraformaldehyde containing PBS for 5 minutes, and analyzed in a fluorescence-activated cell sorter. All clones tested ($n = 60$) were CD4⁺ and expressed the α /beta TCR (data not presented).

LST

LSTs were performed in 96-well U-bottom plates in CM ABS. Each well contained 4×10^4 T cells, which had not been stimulated with antigen or aCD3 for 14 to 18 days, and 4×10^4 autologous EBV-B cells as APCs. The APCs (CFB4:2 for the CFTS4 clones; CFB6:2 for the CFTS6 lines) were, before cocultivation with T cells, incubated overnight with antigen (optimal results were obtained with *Dpt*, 500 μ g/ml, although positive results were also obtained with 5 to 50 μ g/ml) in CM FCS. Excess of antigen was removed by the washing of APCs twice (250 g for 10 minutes at RT). Subsequently, they were irradiated (4000 rad). Every incubation was performed in triplicate. After 4 days, 1 μ Ci of ³H-thymidine was added per well, and incorporation was measured 16 hours later.

Results are presented as net counts per minute; counts per minute of (preincubated) APCs with or without additional rIL-2 and/or rIL-4 were subtracted.

Mitogenicity of the *Dpt* preparation was excluded by demonstrating that T-cell clones from the same and other patients ($N = 6$), with specificity for cat allergen ($N = 3$), GP ($N = 15$), or unknown specificity ($N = 60$) did not respond to *Dpt* with or without addition of exogenous IL-2 and/or IL-4. Moreover, *Dpt*, presented by allogenic EBV-B cells, failed to induce a positive response to *Dpt* in the clones presented here, indicating HLA restriction of the response and excluding mitogenicity of the *Dpt* preparation (data not presented).

Cytokine release

T cells, 10^6 , were stimulated with immobilized aCD3 in 1 ml of CM ABS supplemented with PMA (10 ng/ml) or with 10^6 antigen-preincubated APCs. In the antigen-stimulation experiment, a control stimulation with 10^6 nonpreincubated APCs was performed. Stimulation was performed in a 24-well plate, and after 24 hours of cultivation at 37°C, supernatants were collected and stored at -70°C.

Measurement of cytokines in T cell clone supernatants

Details of assays used for quantitation of IL-2 and IL-5 in supernatants of T-cell clones have been previously pub-

lished.^{15, 16} Briefly, quantitation of IL-2 was measured by adding 0.2 ml of supernatant at different concentrations (1:2 and 1:40) to 4×10^3 indicator cells (CTLL-2 murine cell line). A semiquantitative estimate of IL-2 produced by each T-cell clone was obtained by a standard curve with rIL-2. For the measurement of IL-5, the murine LyH7.B13 cell line was used as a source of indicator cells (kind gift of Dr. R. Palacios, Basel, Switzerland).¹⁷ A semiquantitative estimate of IL-5 produced was obtained by a standard curve with human rIL-5.¹⁸

IFN- γ was determined by ELISA (EASIA ELISA kit, Medgenix Diagnostics, Fleurus, Belgium) according to the manufacturers' recommendations. Supernatants were diluted 1:5, resulting in a detection limit of 5 U/ml. IL-4 and GM-CSF were quantitatively determined by noncommercial sandwich ELISA. GM-CSF: A 96-well flat-bottom plate was coated with 0.1 ml per well (5 μ g/ml, in 20 mmol/L of carbonate buffer) of monoclonal mouse anti-human GM-CSF (F1-1-113-4-6) overnight at 4°C. Blocking was performed with PBS containing 2% bovine serum albumin (dilution buffer) for 2 hours at 37°C, and wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer). Samples and standard preparation (25 ng/ml) were diluted twofold in dilution buffer, added to the wells (0.1 ml), incubated for 2 hours at 37°C, and washed three times with washing buffer. Wells were then incubated with 0.1 ml (1 μ g/ml in dilution buffer) of biotinylated sheep antihuman GM-CSF for 2 hours at RT and washed three times with washing buffer. Incubation followed with 0.1 ml (1:8000 in dilution buffer) of AP-strept for 30 minutes at RT, and washing was performed three times with washing buffer. Finally, 0.1 ml of substrate was added, incubation was performed 20 minutes at RT, and the reaction was stopped with 0.05 ml of 3N NaOH. IL-4: A 96-well flat-bottom plate was incubated with 50 μ l per well (3 μ g/ml in PBS/NaN₃) of monoclonal a-IL-4 (1-41-1) overnight at 4°C and washed four times in washing buffer. Blocking was performed with 0.25 ml of dilution buffer for 3 hours at RT followed by four washes in washing buffer. The rIL-4 standard was diluted from 3.3 ng/ml to 0.01 ng/ml (with 3.3-fold dilutions). Supernatants were added undiluted and 1:5 diluted. Incubation occurred overnight at RT, and the plate was washed four times with washing buffer. Then, 50 μ l (3 μ g/ml in dilution buffer) of monoclonal biotinylated a-IL-4 (1-38-10) was added, and the plate was incubated for 4 hours at RT and washed four times with washing buffer. Incubation followed with 50 μ l (1:1000 in dilution buffer) of AP-strept for 1 hour at RT and washing was performed four times with washing buffer. Finally, 0.05 ml of substrate was added, incubation was performed for 30 minutes at RT, and the reaction was stopped with 0.025 ml 3N NaOH.

Induction of IgE synthesis

Induction of IgE synthesis was performed according to the method of Vercelli et al.¹⁹ In short, 1.5×10^6 mononuclear cells of normal nonatopic donors, isolated as published before,²⁰ were cultured for 9 days with 1 ml of T-cell supernatant in 24-well plates. After 9 days, the supernatants were screened for IgE antibodies with an IgE-specific

ELISA. T-cell supernatants were obtained by stimulating 10^6 T cells with immobilized aCD3 in 1 ml of CM FCS without addition of PMA, in a 24-well plate. After 24 hours of cultivation at 37° C, T-cell supernatants were collected and used immediately or stored at -70° C for later use.

IgE ELISA

A 96-well flat-bottom plate was incubated with 50 μ l per well of GaM (20 ng/ml) for 2 hours at 37° C and washed three times with PBS. Monoclonal a-IgE antibodies (I27) (0.04 μ g/ml in PBS) were incubated for 2 hours at 37° C and washed three times with PBS. Blocking was performed overnight at 4° C with 0.25 ml dilution buffer. After the positive control was washed three times with PBS, the positive control (IgE standard from Boehringer CFTK 92103) and the samples were added to the wells (50 μ l per well), incubated for 3 hours at 37° C, and subsequently washed three times with washing buffer. The IgE standard was diluted from 250 ng/ml to 0.001 ng/ml (with threefold dilutions). Supernatants were added undiluted. Then, 50 μ l of GaH-IgE-biot was added, and the plates were incubated for 2 hours at 37° C and washed three times with washing buffer. Incubation followed with 50 μ l (1:1000 in PBS) of AP-strept for 1 hour at 37° and washing was performed three times with PBS-Tween (0.05%). Finally, 50 μ l of substrate was added, and incubation was performed 30 minutes at RT. IgE concentrations of >0.02 ng/ml were considered positive.

RESULTS

Cytokine release: T cell clones from skin are mainly Th2

The selected CFTS4:2 ($n = 8$) and CFTS4:3 ($n = 7$) clones, as well as the CFTS6:2 and CFTS6:3 lines, were tested for their cytokine profile with an aCD3/PMA stimulation. The results are illustrated in Fig. 1. All clones produced IL-4 and high amounts of GM-CSF (not presented), whereas nine of 12 clones produced IL-5. IL-2, and IFN- γ were only found in the supernatant of clone CFTS4:2.80, which produced 0.5 U/ml of IL-2 and 46 U/ml of IFN- γ , together with IL-4 and IL-5, and may therefore be considered to be a Th0 clone.²¹

Of all clones tested ($n = 30$), 15% appeared to be of Th0 phenotype and 70% of Th2 phenotype. In 15% of the clones, IL-4 was produced in the absence of IL-2, IL-5, or IFN- γ . There was no significant difference between clones derived from the lines CFTS4:2 and CFTS4:3 (in vitro cultured with interleukins or *Dpt* plus interleukins, respectively).

Antigen-specific proliferation is IL-2 dependent

From each cell line raised from skin of patients with AD, at least 50 T cell clones were screened for *Dpt* specificity in an LST. In several repeated tests, about 50% of the clones demonstrated a low, but significant,

proliferation of >1000 cpm over background (Fig. 2, A). Higher antigen concentrations or longer antigen preincubation periods had no amplifying effect (data not presented). Therefore, *Dpt*-specific proliferation assays were repeated with the lines and the selected CFTS4:2 and CFTS4:3 clones in the presence of rIL-4 and/or rIL-2. In Fig. 2, B and C, all clones responded to *Dpt* in the presence of external interleukins. Because of different activation states of the various clones, the background stimulation of the T cells in the presence of rIL-2 varied considerably; however, net stimulations were significant and reproducible in all cases. No significant differences were found between clones derived from the lines CFTS4:2 and CFTS4:3 and the lines CFTS6:2 and CFTS6:3. Control antigens, like GP, did not induce a proliferative response with or without additional exogenous interleukins (data not presented).

Cytokine release after antigen stimulation

After aCD3 plus PMA stimulation, all clones produced high levels of IL-4, a Th2-specific cytokine, as well as GM-CSF, a nonspecific T-cell cytokine, in 85% of the cases without detectable IL-2 or IFN- γ . These results were confirmed with antigen-specific stimulation. In Fig. 3, in all clones, GM-CSF and IL-4 are abundantly produced after antigen-specific stimulation. IL-2 or IFN- γ was not detectable in these supernatants. The clone, CFTS4:2.80, which, on aCD3/PMA stimulation, appeared to be a Th0 clone, failed to produce IL-2 or IFN- γ in several repeated tests. Clones CFTS4:2.6 and CFTS4:2.80 produced only borderline quantities of IL-4 after *Dpt* stimulation; however, antigen specificity could be confirmed by high levels of GM-CSF in the same supernatants of these clones. There was no significant difference between clones derived from the lines CFTS4:2 and CFTS4:3.

Induction of IgE synthesis in normal B cells

Functional tests were performed with 11 of the selected clones. In Table II, induction of IgE synthesis by B cells from normal, nonatopic donors is presented. Supernatants of all clones tested induced IgE production by nonatopic B cells.

DISCUSSION

In this article, we describe allergen-specific, skin-derived T-cell lines and clones from two patients with AD (CF6 and CF4, respectively). The T-cell lines and clones are derived from punch biopsy specimens of positive EPT sites, which were induced with *Dpt* for 24 hours. All lines and clones obtained were CD4⁺ and expressed the alpha/beta TCR. The T-cell cloning procedure we used was slightly different from the

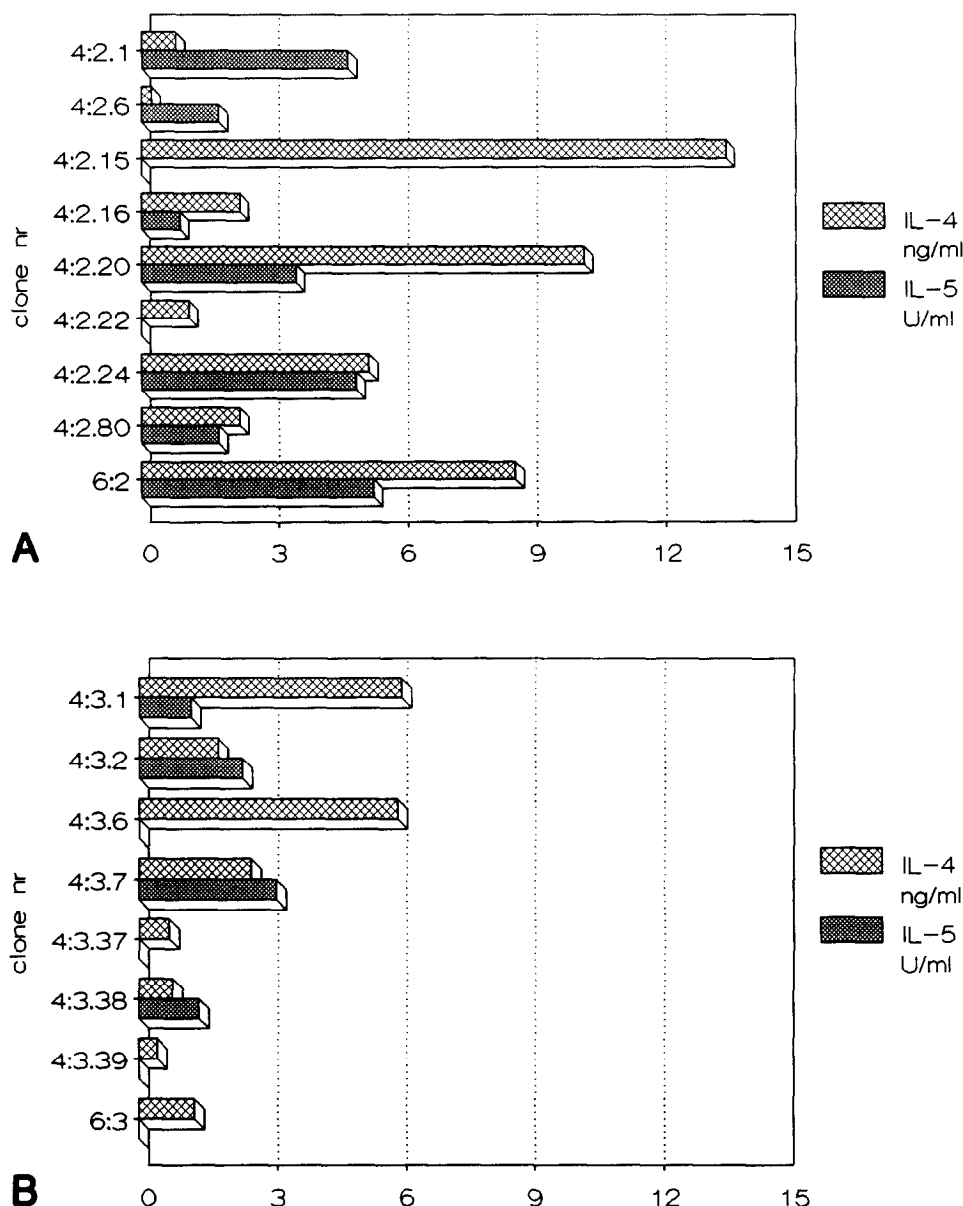


FIG. 1. Cytokine profile of skin-derived T-cell lines and clones. Cells were tested for cytokine release after aCD3/PMA stimulation. All lines and clones produced IL-4, whereas 10 of 15 clones and line CFTS6:2 produced IL-5. No IL-2 or IFN- γ was found in several repeated tests. *CFTS4:2.80 produced additional IL-2 (0.5 U/ml) and IFN- γ , 46 U/ml. **A**, Clones and lines derived from well containing IL-2 plus IL-4. **B**, Clones and lines derived from the well containing IL-2 plus IL-4 and *Dpt*.

generally used protocols. Mitogens were not used, and rIL-4 was added to allow IL-4-dependent T cells to grow and avoid selection of IL-2-responsive T cells.^{22, 23} Furthermore, allogenic EBV-B cells were used as feeders in the subcloning.¹³ No polyclonal, in vitro activation was performed, but the T cells proliferated spontaneously in the wells containing rIL-2 and rIL-4.

Interestingly, we found that the clones from patient

CF4 did not proliferate (with or without additional rIL-2) in response to the feeders used in the subcloning phase (data not presented). This finding indicates that the addition of rIL-2 and rIL-4 supported proliferation for at least 42 days, after which we restimulated the clones in vitro with aCD3 plus rIL-2 and rIL-4. These data confirm and extend recent observations by Reinhold et al.²⁴ who described that T cells from skin of patients with active AD could be grown without re-

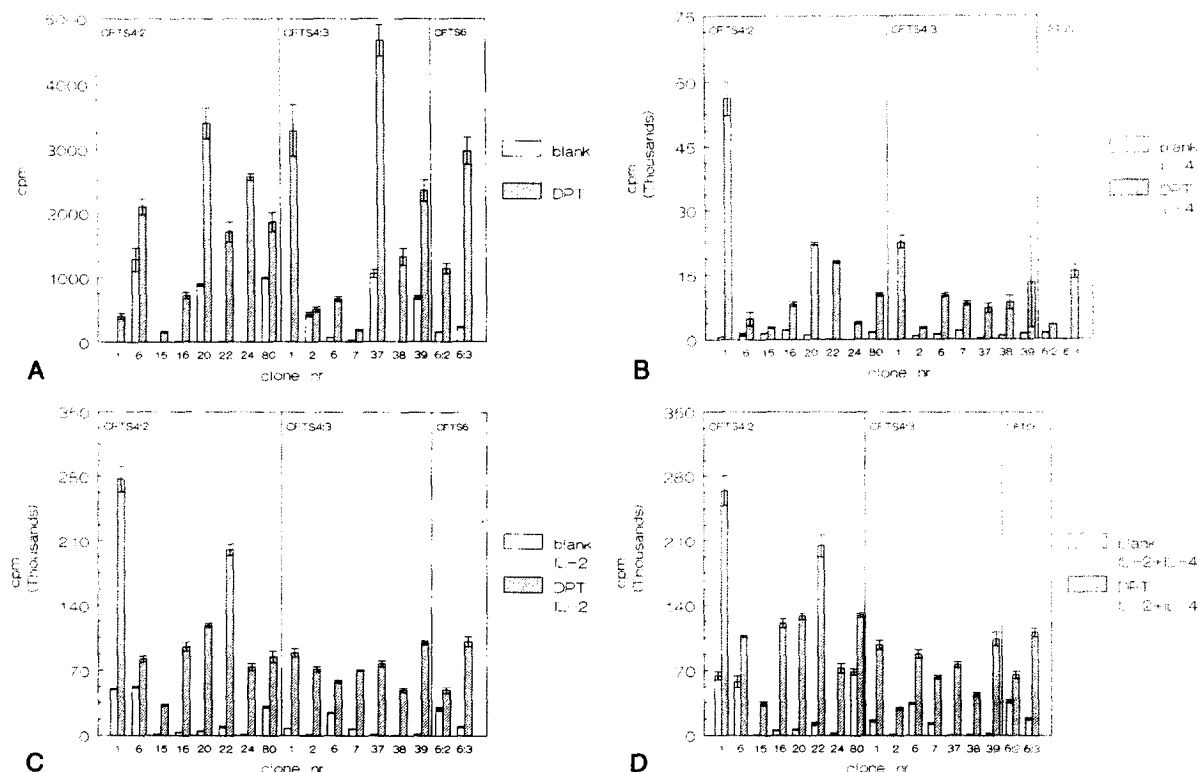


FIG. 2. Allergen-specific proliferation in presence or absence of exogenous IL-2 and/or IL-4. Results are presented as mean $\pm$ SD counts per minute of triplicate cultures. Counts per minute produced by irradiated APC plus/minus allergen plus/minus exogenous interleukins are subtracted. **A**, Antigen-specific proliferation to *Dpt*. **B**, Antigen-specific proliferation to *Dpt* in the presence of IL-4 (50 U/ml). **C**, Antigen-specific proliferation to *Dpt* in the presence of IL-2 (50 U/ml). **D**, Antigen-specific proliferation to *Dpt* in the presence of IL-2 plus IL-4 (both, 50 U/ml).

stimulation in vitro for 21 days in the presence of rIL-2 (50 U/ml) and rIL-4 (1000 U/ml). Clearly, IL-4 may be an essential (co)growth factor for skin T cells. In vitro, in a 5-day culture period, IL-4 (50 U/ml) was more than six times less efficient than IL-2 (50 U/ml) in supporting *Dpt*-specific proliferation, whereas the combination of IL-2 plus IL-4 was comparable to IL-2 alone. In contrast to these results, IL-2 alone (50 U/ml) added to minced skin biopsy specimens did *not* support T-cell growth from positive EPT sites with or without addition of antigen ($n = 7$) (data not presented). We therefore hypothesize that IL-4 functions as a factor that prolongs the responsiveness of skin T cells to IL-2 rather than as a growth factor per se.

Recently, data obtained in mice indicate that the addition of IL-4 (200 U/ml) to polyclonal *naive* T cells in vitro would commit them to Th2 effector cells.^{22, 25} We have tried to rule out this possibility by cloning the T cells from positive EPT sites with IL-2 alone. Unfortunately, we did not succeed. This negative result is in agreement with mouse data that dem-

onstrate that committed Th2 cells are short-lived when they are grown in IL-2 alone.²² Recently, Paliard et al.²⁶ have cloned human T cells from peripheral blood of normal donors, using IL-4 alone or IL-2 alone. In both cases, Th0 cells were found, indicating that IL-4 did not select nor create Th2 cells. We have performed similar experiments using IL-2 plus IL-4 to clone T cells from blood of three patients with AD. Only Th0 cells were found (data not presented).

The T-cell lines and clones described in this article were obtained from skin by adding interleukins with or without *Dpt*. Addition of *Dpt* (50 μ g/ml) to the wells did not result in higher cell numbers (before subcloning) nor in a higher cloning efficiency (after subcloning). Remarkably, after subcloning the T-cell lines from patient CF4, it was found that all clones obtained were specific for the allergen used in the patch test. This included the clones from the line CFTS4:2, which were obtained by addition of IL-2 plus IL-4 alone. Since these T-cell clones were obtained without in vitro polyclonal (re)stimulation with, for example, phytohemagglutinin or allergen, we may

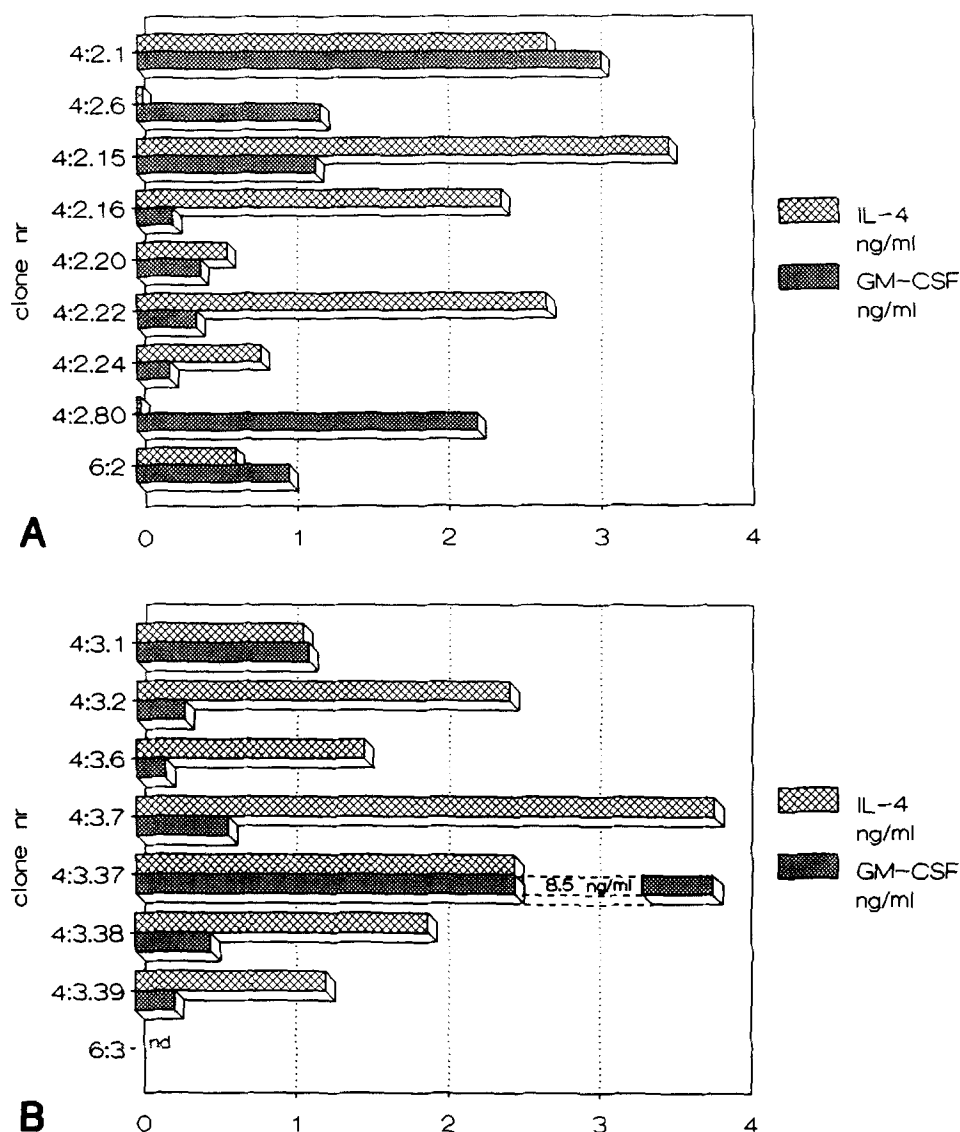


FIG. 3. Cytokine release by T-cell lines and clones after antigen stimulation. IL-4 and GM-CSF release after antigen stimulation are measured. Results are presented as net cytokine production. (There was no detectable interleukin production after incubation with nonpreincubated APCs.) **A**, Clones and lines derived from well containing IL-2 plus IL-4. **B**, Clones and lines derived from well containing IL-2 plus IL-4 and *Dpt*.

conclude that in the 24-hour patch test of this patient, all (in vivo) activated T cells, which we were able to clone, were specific for the allergen. Remarkably, we did not succeed in growing T cells from 48-hour EPT biopsy specimens using IL-2 and IL-4 alone in these two and three other patients. In contrast to this finding, T-cell clones from these patients ($n = 5$) could be obtained with in vitro restimulation with the antigen (manuscript in preparation²⁷). The clones derived from the line CFTS4:3 confirmed that no bystander T cells were activated at 24 hours after EPT. However, in this line, addition of *Dpt* in vitro to the cells may have preferentially expanded antigen-specific T cells.

Whether resting nonspecific (bystander) T cells are present in a 24-hour EPT site cannot be concluded from our data. We realize that in vitro expansion of T cells before subcloning may select for a small number of reactive cells that are further expanded during subcloning, reducing the number of genetically different T-cell clones. In contrast, the constant and reproducible cytokine patterns of the various T-cell clones after allergen or aCD3 plus PMA stimulation do not indicate a clonal relationship between all the clones. Clearly, DNA analysis of the TCR rearrangements will be necessary to solve this point.

Our results demonstrate that in AD aeroallergens

TABLE I. Patients' characteristics

Patient	CF4	CF6
Sex	M	M
Duration of AD	2 yr	>5 yr
Sites of eczema	Face	Face
	Neck	Neck
	Arms	Arms
	Trunk	Legs
Allergic asthma	Yes	Yes
Allergic rhino- conjunctivitis	Yes	Yes
Total sIgE level (kU/L)	1340	6570
GP-specific sIgE (kU/L)	<10	>100
Dpr-specific sIgE (kU/L)	38.4	>100
GP prick test (20 minutes)	Neg	Pos
Dpr prick test (20 minutes)	Pos	Pos
GP patch test (24/48 hours)	Neg/Neg	Pos/Pos
Dpr patch test (24/48 hours)	Pos/Pos	Pos/Pos

sIgE, Serum IgE; Neg, negative; Pos, positive.

may penetrate the skin and induce an allergen-specific T-cell response. This is in agreement with recent data by other groups^{28, 29} who used T cells (lines and clones, respectively) derived from skin from patients with active AD, which, in vitro, responded to GP and to *Dpr*, respectively. Important for the pathogenesis of AD was the finding that most of the T-cell clones derived from skin of patient CF4 appeared to be of Th2 phenotype. Only 15% of the clones produced IL-2 (>0.1 U/ml) and/or IFN- γ (>5 U/ml) (together with IL-4 and IL-5), whereas another 15% of the clones only produced IL-4 without detectable IL-5 (>0.1 U/ml). After allergen-specific stimulation, no IL-2 or IFN- γ was found in several repeated tests. Interestingly, supernatants of all clones tested (including the Th0 clone CFTS4:2.80) were able to induce IgE synthesis in normal B cells in the same range as rIL-4 (50 U/ml), confirming their possible role in the pathogenesis of AD. The T-cell lines derived from positive patch test site of patient CF6 confirmed these findings.

Allergen specificity of the T-cell lines and clones was demonstrated with two independent methods. The classically used proliferation assay proved to be cumbersome and time consuming. Only minor responses were observed in the absence of exogenous interleukins. Addition of IL-2 and/or IL-4 significantly enhanced specific responses, but background responses

TABLE II. Induction of IgE synthesis in normal B cells

Clone no.	IgE (pg/ml)
4:2.1	398
4:2.6	80
4:2.15	123
4:2.16	131
4:2.20	97
4:2.22	136
4:2.24	387
4:2.80	346
4:3.1	76
4:3.6	127
4:3.38	97
4:3.39	180
rIL-4 50 U/ml	572

IgE production was induced in B cells from normal nonatopic donors. Results are presented as net IgE over background.

were often also increased. However, detection of cytokine release after antigenic stimulations proved to be a far better and faster screening assay for antigen specificity than the usually used proliferation assays.³⁰ It is unclear whether the IL-2 dependency to proliferate on antigen-specific stimulation is a feature of T cells derived from skin of patients with AD in general or whether it is a feature of human Th2 T cells. The results obtained by Del Prete et al.¹⁵ argue against the latter. However, it still holds that most investigators select their antigen-specific clones in a proliferative assay without addition of interleukins, which may lead to selection of clones that are independent of exogenous IL-2. Indeed, the clones described by Del Prete et al.¹⁵ are the first human T-cell clones with a stable clear-cut Th2 profile of cytokine production. Mostly, Th2-like cells are reported.^{29, 31, 32} These T-cell clones are functionally Th2, but they are still producing small amounts of IL-2 and/or IFN- γ . In contrast, we hypothesize that IL-2 dependency for proliferation may indeed be a special feature of Th2 (Th2-like) T cells from skin. In contrast to other groups,^{28, 29} we have maintained the infrastructure of the skin for as long as possible, and T cells were grown from the biopsy specimens without the need of additional feeder cells. Therefore, processes that may have started in vivo were allowed to continue for a prolonged, though undetermined, period of time. We have indications²⁷ (manuscript in preparation) that the local environment of the skin is responsible for the fact that skin-derived T cells are dependent on exogenous growth factors for allergen-specific proliferation. This exogenous growth-factor dependency is not observed when the skin T cells are isolated in a single cell suspension and immediately transferred into a blood environ-

ment.^{28, 29} The local environment of the skin may also be responsible for the slightly different (Th2) interleukin pattern that is found in our clones.

In conclusion, allergen applied to the skin of patients with AD induces a T-cell infiltrate of which at least the activated T cells prove to be allergen specific. After cloning, these T cells appear to be of Th2 phenotype on the basis of their cytokine profiles induced by antigen-specific stimulation, as well as their property to induce IgE in normal B cells. After aCD3 plus PMA stimulation, still 85% of the clones produce Th2-specific lymphokines. Allergen-specific Th2 cells may also explain the often very high levels of (specific) serum IgE found in patients with AD. Combining these results with earlier observations that IgE bound to APC (LC) in the skin via their FcR enables these cells to specifically focus and present antigen,^{8, 10} could result in the conclusion that allergen-specific Th2 cells may play an important role in the pathogenesis of AD.

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Leukotriene D₄ receptor blockade inhibits the immediate and late bronchoconstrictor responses to inhaled antigen in patients with asthma

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We have tested the hypothesis that leukotriene D₄ (LTD₄) receptor activation is involved in the development of antigen-induced bronchoconstriction. In two studies, patients with asthma received infusions of placebo or MK-571, a potent and specific LTD₄ receptor antagonist (450 mg or 37.5 mg total dose, respectively). Antigen was inhaled during test-drug administration, and FEV₁ was measured for 10 hours after challenge. Urine samples were collected for measurement of LTE₄; plasma samples were drawn repeatedly for assay of MK-571. MK-571 infusions inhibited both immediate (0 to 3 hours) and late (3 to 10 hours) asthmatic responses. For the high MK-571 dose, the extent of inhibition, as assessed by the area under the curve of FEV₁ versus time was 88% (p = 0.01) and 63% (p = 0.01), for immediate and late responses, respectively. The low MK-571 dose also inhibited both responses but to a minor extent. Mean urinary LTE₄ excretion was elevated after antigen challenge and was unaffected by administration of the LTD₄ receptor antagonist. The present study demonstrates that MK-571 inhibits antigen-induced asthma in a dose-related fashion; it had no effect on antigen-induced increases in urinary LTE₄ excretions. The results suggest that LTD₄ receptor activation plays an important role in antigen-induced asthma. (J ALLERGY CLIN IMMUNOL 1992;90:193-201.)

Key words: Leukotrienes, bronchial provocation tests, asthma, early asthmatic response, late asthmatic response

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Many patients with asthma are atopic and develop airway obstruction on inhalation of patient-specific antigens.¹ When isolated lung tissue from atopic patients with asthma is challenged with antigen, the sulfidopeptide leukotrienes LTC₄, LTD₄, and LTE₄ are released.^{2,3} LTD₄ receptors have been found in large airways and lung tissue homogenates^{4,7}; in man not only LTD₄ but also LTC₄ and LTE₄ appear to interact with these receptors.⁸ These leukotrienes can cause smooth muscle contraction,⁹ increased mucus production,¹⁰ and increased vascular permeability,¹¹ all