

Allergens, IgE, mediators, inflammatory mechanisms

Decreased frequency of interferon- γ - and interleukin-2-producing cells in patients with atopic diseases measured at the single cell level

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Background: Recently, diminished interferon- γ (IFN- γ) and increased interleukin (IL)-4 production in peripheral blood mononuclear cells (PBMCs) from atopic patients have been described by several groups, measured as total cytokine content in culture supernatants. These studies suggested a predominance of T_{H2} -like cells producing large amounts of IL-4 in atopic patients. It is not clear whether the reported cytokine imbalances are the result of an alteration in the distribution of specific T-cell subsets or whether intrinsic dysregulation in cytokine production is a characteristic of atopic individuals.

Objective: This study examined the production of IFN- γ , IL-4, and IL-2 in PBMCs from atopic patients at the single cell level with the use of freshly isolated lymphocytes.

Methods: We recently described a flow cytometric assay in which three-color analysis was used to study the production of a cytokine of interest in a T-cell subpopulation defined by two cell surface markers. PBMCs from 23 atopic patients and 14 control subjects were stimulated with phorbol ester and ionomycin for 5 hours. PBMCs from seven patients and seven control subjects were also cultured with immobilized anti-CD3 antibodies for 24 hours. Cells were fixed, made permeable, and stained for intracellular cytokines in combination with cell surface markers CD3, CD8, and CD45R0. Cytokine-producing cells were analyzed by gating on T-cell subsets.

Results: IFN- γ -producing cells were significantly decreased ($p < 0.05$) in CD4+ T cells but not in CD8+ T cells of atopic patients. CD45R0+ and CD45R0- T cells showed a decreased proportion of IFN- γ -producing cells ($p < 0.05$ and $p < 0.01$, respectively). IL-2 production was diminished in all T-cell subsets ($p < 0.01$). The number of IL-4-producing cells was not elevated, and such cells were exclusively found in the CD45R0+ T cells. Analysis of culture supernatants of sorted CD45R0+ T cells for IL-4 and IFN- γ production confirmed these results.

Conclusion: Our findings provide evidence that a reduced IFN- γ production in atopic patients is due to an intrinsic defect selectively found in the CD4+ T cells. Because IL-2 production was markedly decreased but IL-4 production was unchanged, our data demonstrate a deficiency in the ability of atopic T cells to produce T_{H1} -like cytokines on stimulation with phorbol ester, ionomycin, or anti-CD3 monoclonal antibodies. (*J ALLERGY CLIN IMMUNOL* 1995;96:515-27.)

Key words: Atopy, T cell, cytokine, single cell assay, flow cytometry

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Abbreviations used

AD:	Atopic dermatitis
bp:	Base pairs
FITC:	Fluorescein-isothiocyanate
HBSS:	Hanks' balanced salt solution
HEPES:	N-s-hydroxyethylpiperazine-N-2-ethanesulfonic acid
IFN:	Interferon
IL:	Interleukin
MTT:	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
PHA:	Phytohemagglutinin
PBMCs:	Peripheral blood mononuclear cells
PMA:	Phorbol 12-myristate 13-acetate
RT-PCR:	Reverse-transcriptase polymerase chain reaction

During the last few years it has become increasingly clear that T cells contribute to the abnormal regulation of the immune response in atopic diseases. A number of phenotypic alterations, reduced proliferative responses to mitogens and recall antigens, and an impaired cytotoxicity have been described.¹ Because T cells are potent producers of a large variety of cytokines, studies were undertaken to investigate cytokine production in atopic disorders. Allergen-specific T-cell clones, raised from the blood or inflamed skin of atopic patients, produced either a T_{H2} cytokine pattern (interleukin [IL]-4 and IL-5) or a T_{H0} pattern (interferon- γ [IFN- γ], IL-2, IL-4, and IL-5).²⁻⁴ Furthermore, only T-cell clones producing IL-4 were shown to induce IgE synthesis in vitro.⁵ On polyclonal stimulation, peripheral blood mononuclear cells (PBMCs) from patients with atopic dermatitis (AD) produced lower amounts of IFN- γ ⁶⁻⁹ and higher amounts of IL-4⁷⁻¹⁰ when compared with PBMCs from healthy control subjects. Taken together, these results suggested a pool of T_{H2} -like-reactive T cells in atopic patients.^{1, 11, 12} However, whether a clear dichotomy in T_{H1} and T_{H2} cells exists in vivo remains unknown because of the failure of appropriate single cell assays.

The subdivision of T-helper cells by CD45 isoforms revealed two functionally different subsets.¹³ CD4⁺ CD45RA⁺ "naive" cells show a limited functional capacity to respond to antigens and to produce cytokines including IFN- γ , IL-4, and IL-5. After in vivo or in vitro priming, these cells gain the ability to produce a variety of cytokines and to respond to antigens. During this maturation, cells lose the CD45RA isoform and ex-

press the CD45R0 isoform. Hence, CD45RA⁺ (CD45R0⁻) naive cells are potential precursors for CD45R0⁺ (CD45RA⁻) "memory" T cells.¹⁴ Cells with a naive phenotype were found to be enhanced in atopic children and exhibited a delay in switching to the memory cell phenotype with age.¹⁵ Such an imbalance in CD45RA⁺ and CD45R0⁺ T cells could underlie the reduced total IFN- γ production in mononuclear cells from atopic patients. As a result, assays measuring the total cytokine content in culture supernatants of stimulated heterogeneous cells may not be helpful in distinguishing between cytokine imbalances caused by an altered composition of regulatory T cells or an intrinsic defect in cytokine production.

We recently described a single cell assay system to detect intracellular cytokines with three-color flow cytometry. This enabled us to measure a particular cytokine of interest in a defined cell population or to examine the coexpression of two different cytokines.¹⁶ This technique takes into account different capacities of cells to produce a particular cytokine. In this study we show that it is predominantly the CD4⁺ and CD45R0⁺ T-cell subset from atopic patients that had a reduced capacity to produce IL-2 and IFN- γ , whereas the number of cells producing IL-4 was not enhanced. ELISA analysis of supernatants from isolated CD45R0⁺ T cells confirmed these findings. We therefore suggest that CD45R0⁺ memory T cells from atopic patients are impaired in their production of IFN- γ and IL-2.

METHODS**Antibodies and reagents**

Anti-IFN- γ (mouse-IgG₁ GZ-4 [Boehringer Mannheim, Germany], mouse-IgG_{2a} [Genzyme, Boston, Mass.]), anti-IL-2 (mouse-IgG_{2a}, N7.48A) kindly provided by A. Jurado (Sandoz, Basel, Switzerland), anti-IL-4 (mouse-IgG₁, 8F12, 4D9, 1G1), and anti-IFN- γ (mouse IgG₁, 45.15) generated at Ciba Geigy, Basel and kindly provided by C. Heusser were used.¹⁶ All three anti-IFN- γ antibodies showed a similar staining profile. Specificity of the anti-cytokine antibodies was tested earlier.^{16, 17} Furthermore, Western blotting studies showed that each antibody bound only the specific antigen. Isotype-specific goat-anti-mouse IgG₁ or IgG_{2a} second-step antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin were obtained from Southern Biotechnology (Birmingham, Ala.). All second-step antibodies were tested for specificity; cross-reactivity was below 1%. Control antibodies for IgG₁ and IgG_{2a} were purchased from Coulter (Krefeld, Germany). Antibodies against surface antigens labeled with FITC, phycoerythrin, or peridinin-chlorophyll were purchased from Becton Dickinson (Heidelberg, Ger-

TABLE I. Clinical features of the patients for parts 1 and 2 of the study

	Patient No.	Age (yr)	Diagnosis	Severity*		Medication†
				Asthma	AD	
<i>Part 1</i>	1	12.5	Asthma	3		Theophylline, steroids (400-800 µg/day), DSCG
	2	10.0	Asthma	2		Steroids (400-800 µg/day), DSCG
	3	11.2	Asthma	2		Theophylline
	4	10.3	Asthma	4		Theophylline, steroids (600-1200 µg/day), DSCG
	5	8.7	Asthma, AD	1	Low	LO
	6	11.3	Asthma, AD	1	Mod	LO
	7	7.8	Asthma, AD	1	Low	LO
	8	15.3	AD		Mod	LO
	9	20.0	AD		Sev	Topical steroids
	10	28.4	AD		Sev	Topical steroids
	11	23.1	AD		Sev	LO
	12	2.5	AD		Sev	LO
	13	14.7	AD		Mod	LO
	14	12.0	Asthma, AD	3	Low	Theophylline, steroids (1200 µg/day)
	15	13.0	Asthma, AD	3	Mod	Theophylline, steroids (800 µg/day)
						Topical steroids
	16	12.0	Asthma	5		Theophylline, steroids (1200 µg/day)
	17	12.0	Asthma	4		Theophylline, steroids (800 µg/day)
	18	11.0	Asthma	3		Steroids (800 µg/day)
	19	7.0	Asthma	3		Steroids (800 µg/day)
	20	13.0	Asthma	4		Theophylline, steroids (600 µg/day)
	21	10.0	Asthma	3		Theophylline, DSCG
	22	9.0	Asthma	3		DSCG
	23	10.0	Asthma	4		Steroids (800 µg/day)
<i>Part 2</i>	1	31	AD, Asthma	1	21	LO
	2	54	AD		70	LO
	3	18	AD		49	LO
	4	58	AD		69	LO
	5	20	AD		49	LO
	6	15	AD		55	LO
	7	44	AD		50	LO

DSCG, Disodium chromoglycate; LO, lubricating ointment.

*Severity was calculated according to scores as previously described^{19,20}: patients with asthma were graded from 1 (lowest) to 5 (highest); patients with AD were graded from 0 to 100 (highly severe). In the first part of the study, patients with AD were characterized as having low, moderate (Mod), or severe (Sev) disease activity.

†Patients with asthma used inhaled β₂-agonists if required; steroids (beclomethasone) were given exclusively by inhalation.

many): anti-CD4, anti-CD8, anti-CD45R0, anti-CD45RA, anti-CD3. For the second part of the study anti-cytokine antibodies were coupled to FITC with a standard procedure (Molecular Probes, Eugene, Ore.). Recombinant cytokines were obtained from Genzyme.

Phorbol 12-myristate 13-acetate (PMA), ionomycin, monensin, salbutamol, dexamethasone, and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Deisenhofen, Germany). Phytohemagglutinin (PHA) was obtained from Biochrom (Berlin, Germany), saponin and paraformaldehyde were from Riedel de Haen (Seelze, Germany), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer was from Serva (Heidelberg, Ger-

many), and Hanks' balanced salt solution (HBSS) buffer was purchased from Biochrom.

Patients and control subjects

Peripheral blood was obtained from 23 atopic patients (aged 2.5 to 28.4 years; median age, 12.3 years) and 14 healthy control subjects (aged 1.4 to 29.5 years; median age, 14.7 years). Patients with AD were diagnosed according to the criteria of Hanifin.¹⁸ The severity of AD in the second part of the study (seven patients) was calculated with the Costa score.¹⁹ The diagnosis of asthma was made using the criteria of the American Thoracic Society; the severity was assessed as described previously.²⁰ Table I summarizes the clinical features of

all patients. None of the patients were treated with systemic steroids. Patients with asthma were treated, depending on the severity of the disease, with inhaled salbutamol when required, disodium chromoglycate, beclomethasone, and/or oral theophylline. Three patients with AD received topical glucocorticoids; all others received only lubricating ointments. None of the patients had acute exacerbation of their disease. Written consent was obtained from the parents of children younger than 18 years of age. Patients had a family history of atopy (18 of 23), elevated serum IgE, and detectable specific IgE antibodies to inhaled allergens as measured with the Phadiatop test (Pharmacia, Freiburg, Germany). Peripheral blood was obtained from three healthy volunteers for *in vitro* treatment with salbutamol or dexamethasone. Two healthy adult volunteers were treated with inhaled beclomethasone, 600 µg/day, for 7 days. Cytokine production of PBMCs was investigated 2 days before the study began, on the day the experiment started, and after 3 and 7 days.

Cells and cell culture

Mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Biochrom). CD45RA⁺ or CD45R0⁺ T-cell subpopulations were isolated as described previously.¹⁶ A combination of sheep red blood cell isolation followed by magnetic activated cell sorter depletion permitted the isolation of highly purified T-cell subsets (>97%). PBMCs or purified T cells were cultured in 96-well flat-bottom plates (2×10^5 cells per 100 µl) or in 24-well plates (2×10^6 cells/ml) (Nunc, Wiesbaden, Germany) for 5 hours at 37°C with 8% CO₂ in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2×10^{-5} mol/L mercaptoethanol (Biochrom), and 10% fetal calf serum (Sigma). Cells were stimulated with 10 ng/ml PMA + 1 µmol/L ionomycin in the presence of 1 µmol/L monensin for 5 hours. Additionally, in the second part of the study, cells were stimulated via the CD3 complex with immobilized anti-CD3 antibodies (OKT3, 20 µg/ml, kindly provided by C. Heusser) for 24 hours. Monensin was added 15 hours before cells were harvested.

Cell staining and analysis were accomplished as described previously.¹⁶ Briefly, cultured cells were washed twice in HBSS and then fixed in ice-cold HBSS containing 4% paraformaldehyde for 10 minutes. After two additional washes in HBSS, cells were resuspended to a concentration of 2×10^5 cells. Cytokine-specific antibodies diluted in HBSS with 0.1% saponin and 0.01 mol/L HEPES buffer (saponin-buffer) were added at a concentration of 1 µg/ml for 30 minutes at room temperature. Cells were washed twice in saponin-buffer and subsequently incubated with isotype-specific second-step antibodies at concentrations ranging from 0.5 to 5 µg/ml for 20 minutes in the dark. Cells were washed in saponin-buffer, and free binding sites of the second-step

antibody were blocked by incubation with mouse IgG (100 µg/ml, Sigma). Thereafter, cells were incubated with directly conjugated monoclonal antibodies against the cell surface antigens of interest. The blocking procedure was necessary to prevent any cross-reaction between the goat anti-mouse antibody and the antibodies against cell surface markers. To determine whether the blocking procedure was efficient, we compared this indirect method with direct three-color staining by using FITC-conjugated anti-cytokine antibodies. No differences between the two methods were observed.

To ensure that the PBMC isolation procedure did not influence cytokine production, we compared PMA- and ionomycin-activated PBMCs and whole blood cells diluted 1:10 with culture medium. When lymphocytes were gated, no significant difference in frequency of cytokine-producing cells could be observed between the two methods (3 experiments).

IL-2 bioactivity was measured with cytotoxic lymphoid line cells and an MTT assay as described by Denizot and Lang.²¹ Briefly, supernatants were obtained from PHA-stimulated PBMCs from seven atopic and eight healthy children after 24 and 48 hours. Ten thousand cytotoxic lymphoid line cells were cultured with serial dilutions of the supernatants and with recombinant IL-2 to generate a standard curve. After 48 hours of culture, cells were washed, resuspended in RPMI-1640 medium (without phenol red) containing 0.5 mg MTT, and cultured for 4 hours. Thereafter, cells were resuspended in isopropanol and monitored with an ELISA reader (Flow Laboratories) at 570 nm. Results are calculated as IL-2 in units per milliliter produced from 1 million PBMCs.

Flow cytometry

A FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) equipped with a 15 mW argon ion laser and filter settings for FITC (530 nm), phycoerythrin (585 nm), and TRI-Color (Medac, Hamburg, Germany) emitting in deep red (>650 nm) was used. Ten thousand cells were computed in list mode and analyzed with the LYSIS II and PC-LYSIS software (Becton Dickinson). Analysis gates were set on lymphocytes according to forward and sideward scatter properties. Results are expressed as the percentage of cytokine-producing cells in a population of specified T cells.

ELISA

Sorted CD3⁺ CD45RA⁺ and CD3⁺ CD45R0⁺ cells from five healthy donors and five atopic patients were stimulated with PMA and ionomycin in the absence of monensin. Culture supernatants were harvested after 24 hours and tested for secreted IFN-γ and IL-4 with commercially available ELISA kits (Biomar, Marburg, Germany and Genzyme, respectively). The detection limit for IFN-γ was 1 U/ml, and for IL-4, 20 pg/ml. CD3⁺ CD45R0⁺ cells were obtained from 14 healthy and 14 atopic donors and stimulated with PHA (2.4 µg/ml) in the presence of 30% E⁻ cells (monocytes and

B cells obtained after sheep red blood cell rosetting). Supernatants were analyzed after 24 hours for IL-4 by ELISA. For the second part of the study, PBMCs from seven patients and seven healthy control subjects were stimulated with PMA + ionomycin and anti-CD3 antibodies. Culture supernatants were harvested after 24 hours. ELISA analysis was performed with the clones 43.11 (anti-IFN- γ) and 3H4 (anti-IL-4) to capture and clones 45.15-biotin (anti-IFN- γ) and 8F12-biotin (anti-IL-4) to detect the particular cytokine. All antibodies were kindly provided by C. Heusser. Avidin-peroxidase and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) were used as substrate; the detection limit was 10 pg/ml in both systems.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Freshly isolated PBMCs and PMA + ionomycin-activated PBMCs (5 hours) from three atopic patients and three healthy control subjects were tested for mRNA expression of β -actin, IFN- γ , IL-2, and IL-4 according to the protocol described by Ehlers and Smith.¹⁴ Briefly, total RNA was obtained from 10^6 cells and reverse-transcribed, and cDNA corresponding to 10^5 cells was amplified with specific primers. The primers were 5' ctt ccc cct ctg ttc ttc ct 3' and 5' ttc ctg tcg age cgt ttc ag 3' for IL-4 (PCR product, 297 base pairs [bp]), 5' agt tat atc ttg gct ttt ca 3' and 5' acc gaa taa tta gtc agc tt 3' for IFN- γ (PCR product, 336 bp), 5' act cac cag gat gct cac at 3' and 5' agg taa tcc atc tgt tca ga 3' for IL-2 (PCR product, 246 bp), 5' gaa act acc ttc aac tcc atc 3' and 5' cta gaa gca ttt gcg gtg gac gat gga ggg 3' for β -actin (PCR product, 303 bp).

Statistics

Differences between the study groups were tested with the Mann-Whitney nonparametric U test.

RESULTS

Patient medication had no effect on cytokine production

To determine whether patients' medication would influence intracellular IFN- γ production, we performed in vitro tests with varying concentrations of salbutamol and dexamethasone. Salbutamol at a concentration range of 1 to 10,000 ng/ml did not influence the frequency of PMA- and ionomycin-induced IFN- γ -producing cells. (PBMCs from three healthy volunteers were tested.) When PBMCs from healthy donors were stimulated with PMA and ionomycin in the presence of dexamethasone (1 to 1000 ng/ml), a reduction in the number of IFN- γ -producing cells was found: 1000 ng/ml, 40% reduction; 100 ng/ml, 34% reduction; 10 ng/ml, 22% reduction; and 1 ng/ml, 10% (median of 3 experiments). To determine whether inhaled corticosteroids

would cause such a reduction, two healthy volunteers were treated with beclomethasone, 200 μ g, three times a day for 7 days. Blood was obtained 2 days before treatment (d-2), on the day treatment began (d0), on day 3 (d3), and on day 7 (d7) after the inhalation therapy was started. PBMCs were stimulated, and the proportion of IFN- γ -producing cells was examined. In both subjects no significant alterations were found, regardless of the stimulus (PMA + ionomycin or anti-CD3):

Donor 1: PMA + ionomycin d-2 16.7% of PBMCs, d0 14.8%, d3 13.9%, and d7 17.1%,
anti-CD3 d-2 1.6%, d0 1.5%, d3 0.9%, and d7 1.1%.

Donor 2: PMA + ionomycin d-2 14.0% of PBMCs, d0 10.8%, d3 11.2%, and d7 14.0%,
anti-CD3 d-2 0.8%, d0 1.1%, d3 1.5%, and d7 1.4%.

Lymphocytes from atopic patients compared with those from healthy control subjects

Ex vivo isolated cells did not produce IFN- γ or IL-4 as measured by mRNA analysis and our protein staining method. However, all tested samples (three atopic and three healthy) were positive for specific mRNA expression after 5 hours of PMA and ionomycin stimulation (Fig. 1). Therefore to study cytokine expression in atopic T cells, we cultured PBMCs with PMA and ionomycin in the first part of the study and with anti-CD3 antibodies in the second part. The results shown in Table II indicate that within the total lymphocyte population of atopic patients, the proportion of IFN- γ - and IL-2-producing cells was significantly ($p < 0.01$) reduced compared with that of a healthy control group when stimulated with PMA and ionomycin. No differences were found for IL-4-producing cells. To determine whether this finding was stimulus-dependent, we stimulated PBMCs in the second part of the study with immobilized anti-CD3 antibodies. As Table III shows, PBMCs from seven atopic patients produced less IFN- γ and nearly equal amounts of IL-4 during 24 hours of stimulation compared with PBMCs from healthy control subjects. The frequency of IFN- γ - and IL-2-producing cells was reduced in the atopic group. Thus a diminished frequency of IFN- γ - and IL-2-producing cells in PBMCs from atopic

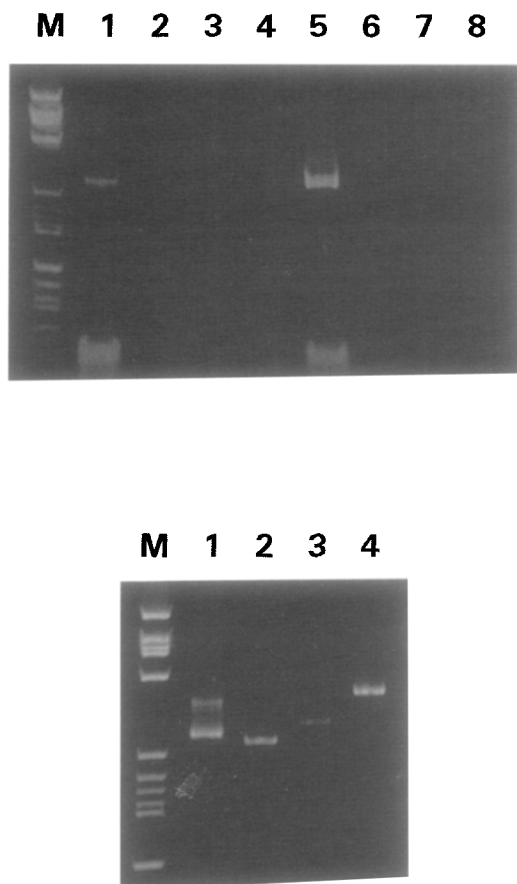


FIG. 1. Top, Prepared total RNA from freshly isolated PBMCs from an atopic donor and a healthy donor were reverse-transcribed, and cDNA was amplified (RT-PCR) with specific primers for β -actin (lanes 1 and 5), IL-2 (lanes 2 and 6), IL-4 (lanes 3 and 7), and IFN- γ (lanes 4 and 8). Lanes 1 to 4 show the results for the atopic donor, and lanes 5 to 8 show the results for the healthy donor. **Bottom,** PBMCs from the atopic donor were stimulated with PMA and ionomycin for 5 hours, and RT-PCR was performed as described above.

patients was independent of the stimulus. When lymphocytes were stained for IFN- γ and IL-4 simultaneously, no differences were observed in the expression of double-positive cells between the two study groups: in all cases the number was lower than 0.5%, even in the one sample in which 11.9% of the lymphocytes were positive for IL-4. A substantial proportion of double-positive lymphocytes for IL-2 and IFN- γ was always found, but it differed between atopic and healthy donors: of all IFN- γ - or IL-2-positive cells, 10% to 30% were positive for both cytokines. In five atopic patients the median for IFN- γ IL-2 double-positive cells was 1.9% (range, 0.5% to 6.2%) of total lymphocytes, and

in five healthy control subjects the median was 5.5% (range, 2.1% to 9.7%).

IL-2-producing cells were significantly reduced in all tested T-cell subsets from atopic patients

When PBMCs were stimulated with PHA and supernatants were analyzed for IL-2, a reduced IL-2 bioactivity was found in the atopic patient group ($n = 7$) compared with a healthy control group ($n = 8$): the median for the patient group was 0.86 U/ml (range, 0.22 to 4.9 U/ml), and the median for the control group was 1.7 U/ml (range, 1.3 to 3.8 U/ml) after 24 hours of culture ($p < 0.05$). After 48 hours, median values were 0.5 U/ml (range, 0.1 to 3.1 U/ml) for the patient group versus 1.9 U/ml (range, 0.3 to 5.0 U/ml) for the control group (no significant difference). To determine which T-cell subpopulation was responsible for this reduced IL-2 activity, we performed flow cytometric three-color staining techniques, which enabled us to define the T-cell subset by two cell surface markers, and the cytokine of interest was measured by using the third color. Thus CD3+, CD3+CD8+, CD3+CD45R0+, and CD3+CD45R0- T cells could be analyzed. Because CD4 expression is downregulated after PMA and ionomycin stimulation,²² the CD3+CD4+ population could only be analyzed by gating on the CD3+CD8- cell population. The frequency of IL-2-producing cells was markedly reduced in all T-cell subsets examined from the atopic patients compared with normal subjects (Table II). When the frequency of IFN- γ -producing cells was examined, a decreased number was found within the CD3+ and CD3+CD4+ cell population, but not within CD3+CD8+ cells. Fig. 2 shows an example in which nearly 50% of the CD3+CD8+ cells produced IFN- γ . IFN- γ -producing cells were identified among both CD45R0+ and CD45R0- T cells, and in each case were significantly lower in frequency than in control subjects. Analysis of IL-4-producing cells showed that virtually all were of the CD3+CD45R0+ phenotype, as shown in Fig. 3, and were detected among CD4+, as well as CD8+ T cells; but no differences were found between atopic patients and normal subjects (Table II).

To confirm the data obtained by gating on T-cell subpopulations in PBMCs, we analyzed culture supernatants for IL-4 and IFN- γ production from sorted, PMA- and ionomycin-stimulated CD45R0+ and CD45RA+ T cells. As shown in Table IV, no differences were found between the groups for

TABLE II. Proportion of cytokine-producing cells in 11 atopic patients and 11 healthy control subjects

Subset	IFN- γ		IL-2		IL-4	
	AP	NAS	AP	NAS	AP	NAS
CD3+	8.2* (1.3-27.0)	15.7 (6.5-39.4)	8.8† (1.5-16.8)	28.9 (5.1-43.8)	1.1 (0.3-8.8)	0.9 (0.1-5.9)
CD3+CD8+	19.6 (2.9-45.8)	19.4 (12.5-35.4)	4.1† (0.5-8.5)	9.8 (3.7-41.8)	0.8 (0-2.6)	0.8 (0.1-2.3)
CD3+CD4+(CD8-)	8.6* (0.8-20.5)	13.4 (5.3-38.3)	11.6† (0.1-20.0)	29.6 (4.5-48.2)	1.5 (0.1-7.6)	1.2 (0.3-5.3)
CD3+CD45R0+	19.4* (5.1-40.2)	27.2 (7.4-37.7)	11.1† (7.3-22.4)	27.9 (11.0-60.6)	2.9 (0.5-10.4)	1.6 (0.8-9.3)
CD3+CD45R0-	4.5† (0.9-14.1)	11.1 (3.1-32.4)	3.4† (0-13.3)	13.8 (1.4-25.1)	0.1 (0-0.7)	0.2 (0-0.6)
Total lymphocytes	7.9† (1.2-25.1) <i>n</i> = 23	18.4 (6.3-38.3) <i>n</i> = 14	9.1† (1.7-17.1) <i>n</i> = 13	23.0 (4.6-41.0) <i>n</i> = 14	1.5 (0.1-11.9) <i>n</i> = 23	1.1 (0-2.4) <i>n</i> = 14

AP, Atopic patients; NAS, nonatopic subjects.

PBMCs were stimulated and stained as described in the Methods section. The proportion of cells producing the particular cytokine is shown as a percentage of the indicated T-cell subpopulation as the median, and the range is given in parentheses. Statistically significant differences between the two groups are shown.

**p* < 0.05.

†*p* < 0.01.

IL-4, but reduced amounts of IFN- γ among atopic CD45R0+ and CD45RA+ T cells were detected. Because phorbol ester and ionomycin stimulation do not require cell surface receptors, we stimulated PBMCs with the mitogen PHA in the presence of monocytes and B cells. Fourteen patients and 14 control subjects were tested. Supernatants of CD45R0+ T cells were analyzed for IL-4 after 24 hours. Again, there was no difference between the two groups (median for patients, 119 pg/ml; range, 32 to 472 pg/ml; median for control subjects, 87 pg/ml; range, <10 pg/ml to 402 pg/ml).

DISCUSSION

To study cytokine production of lymphocytes from atopic patients at the single cell level, we analyzed IFN- γ , IL-2, and IL-4 production by flow cytometry. Freshly isolated lymphocytes from atopic and healthy donors did not produce cytokines as measured by intracellular staining and RT-PCR techniques. However, mRNA for IL-5, IL-4, IL-2, and IFN- γ was found in activated lymphocytes from patients with acute exacerbation of asthma and healthy volunteers,²³ but only when amplified PCR products were detected by radiolabeled internal oligonucleotides, indicating that the amount of specific mRNA was low. In this study only IL-5 mRNA was enhanced when compared with healthy control subjects.²³ This observation

and our results suggest that peripheral lymphocytes even from atopic patients do not contain substantial amounts of in vivo activated cytokine-producing cells.

When peripheral lymphocytes were stimulated in vitro, we could demonstrate a reduced frequency of IFN- γ - and IL-2-producing cells in atopic individuals. We have used phorbol ester and ionomycin stimulation to circumvent membrane receptor-associated signal pathways and anti-CD3 antibodies as a well-defined stimulus through CD3/T-cell receptor signaling. Because reports showing a reduced capacity of PBMC bulk cultures to produce IFN- γ ⁶⁻⁹ failed to identify the target T-cell population, it remains unclear whether these results are due to imbalances in T-cell subpopulations^{1, 15} having different abilities to produce cytokines or uncharacterized intrinsic defects to produce IFN- γ . The subdivision of T cells by CD45 isoform expression reveals two subpopulations differing in cytokine production.^{13, 14} On stimulation, CD45RA+ naive T cells produce IL-2, little or no IFN- γ , and no IL-4. After prolonged in vitro stimulation or in vivo with aging, naive cells gain the ability to produce IFN- γ , IL-4, IL-5, and other cytokines¹⁴; to express the CD45R0 isoform; and to respond to recall antigens, now called "memory" T cells.¹³ Previously, we and others showed that CD45RA+ naive cells were increased

TABLE III. Proportion of cytokine-producing cells and cytokine content in supernatants on anti-CD3 and PMA + ionomycin stimulation

	Patients (n = 7)		Control Subjects (n = 7)	
	% Lymphocytes	Reactivity (pg/ml)	% Lymphocytes	Reactivity (pg/ml)
anti-CD3				
IFN- γ	1.5* (0.8-5.7)	1480 (227-14320)	3.9 (1.7-7.1)	7411 (1696-21995)
IL-4	<0.5 (<0.5-3.6)	197 (32-324)	<0.5 (<0.5-2.1)	421 (115-1039)
IL-2	2.5 (1.0-9.6)	ND	7.0 (1.5-9.7)	ND
PMA + ionomycin				
IFN- γ	4.8† (2.5-14.8)	7741 (800-60890)	17.1 (8.2-22.4)	17268 (7590-68675)
IL-4	0.8 (<0.5-1.9)	177 (75-558)	1.0 (<0.5-6.4)	415 (253-1840)
IL-2	7.1 (3.8-33.5)	ND	17.6 (6.8-22.1)	ND

ND, Not determined.

PBMCs were stimulated with immobilized anti-CD3 antibodies for 24 hours and with PMA + ionomycin for 5 hours as described in the Methods section. For measuring the proportion of cytokine-producing cells, monensin (1 μ mol/L final concentration) was added 15 hours before harvesting (in the case of anti-CD3 stimulation) or directly after PMA + ionomycin stimulation. An analysis gate was set on lymphocytes; results are expressed as proportion of cytokine-producing cells. Supernatants were obtained after 24 hours of culture in the absence of monensin and analyzed for IFN- γ and IL-4 reactivity by ELISA. Results are expressed as median, with ranges in parentheses.

* $p < 0.05$.† $p < 0.01$.

and CD45R0+ memory cells were decreased in peripheral blood from atopic patients,^{15,24} although no differences in relative or absolute numbers of CD4+ and CD8+ T cells were found.¹⁵ An imbalance in CD45RA+ and CD45RO+ cells could explain a reduced IFN- γ production in PBMC bulk cultures from atopic patients. Therefore it was important to determine cytokine production at the single cell level.

Using a flow cytometric single cell assay, we have demonstrated that the proportion of IFN- γ -producing cells is significantly reduced in the CD3+CD45R0+, CD3+CD45R0-, and CD4+(CD8-) cell populations of atopic patients. ELISA analysis of supernatants of these cells confirmed these observations. Our data were obtained after 5 hours of culture with PMA and ionomycin and after 24 hours with anti-CD3 stimulation. We¹⁶ and others¹⁷ have previously shown that PMA- and ionomycin-stimulated T cells produced maximum amounts of IL-2 or IFN- γ after 6 to 10 hours, whereas the number of IL-4-producing cells peaked between 3 and 6 hours. When cells were stimulated with anti-CD3 antibodies, no clear peak was observed

during three time-course experiments: IL-4-producing cells were detected between 10 and 36 hours, and IFN- γ - and IL-2-producing cells were found between 16 and 48 hours. Thus our chosen time points were a compromise. CD4+ T cells were identified as CD3+ CD8- cells because of the downregulation of CD4 after phorbol ester stimulation.²² This assumption is correct, given that CD3+ CD4- CD8- (γ/δ T-cell receptor-positive) cells are not expanded in vivo. We showed earlier that children and adolescents with atopic diseases have a reduced frequency of γ/δ receptor-positive T cells in their peripheral blood when compared with healthy individuals.²⁵ Thus we do not have evidence that our ability to detect CD4+ T cells by analyzing CD3+ CD8- cells is not true. Our data indicate that a diminished IFN- γ production in bulk cultures of atopic PBMCs is not attributable to changes in lymphocyte subset distribution alone. Our results are consistent with the hypothesis of "intrinsic defect" within T cells of atopic patients.⁶ Indeed, a lower basal protein kinase C activity in PBMCs from patients with AD was described.²⁶ Because phorbol esters are potent inducers of

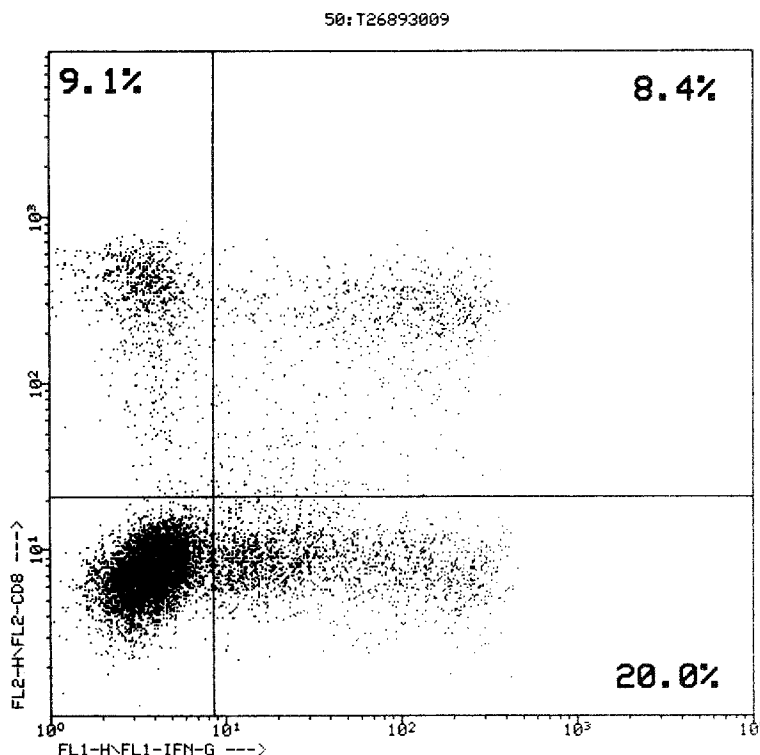


FIG. 2. PBMCs from a healthy donor were stimulated for 5 hours with PMA and ionomycin in the presence of monensin. Cells were stained for IFN- γ (horizontal), CD8 (vertical), and CD3 (third color, not shown). An analysis gate was set on lymphocytes by scatter properties and CD3+ cells. Analysis shows that 17.5% of the cells were CD3+CD8+, dividing into a CD3+CD8+ IFN- γ - (9.1%) and a CD3+CD8+ IFN- γ + population (8.4%). The proportion of IFN- γ -producing cells within this CD3+CD8+ cell population is calculated as 47.8%.

protein kinase C, altered enzyme activities might explain diminished IFN- γ and IL-2 responses in atopic T cells.

Because switching of CD45RA+ naive cells to CD45RO memory cells requires proliferation and because proliferation of T cells is dependent on IL-2,²⁷ an imbalance in T-cell subpopulations in the peripheral blood of atopic patients might be due to insufficient endogenous IL-2 production. A greatly reduced number of IL-2-positive cells was found in this study in all T-cell subpopulations analyzed, but the number was dramatically reduced within CD4+ T cells, which were the most potent T cells to produce IL-2 in normal subjects. This result is in accordance with our finding of a reduced IL-2 bioactivity in supernatants of PHA-stimulated PBMCs obtained from seven atopic patients. A diminished proliferative response of atopic T cells to mitogens¹ or anti-T-cell receptor/CD3 antibodies is a common feature of these cells and may be due to a reduced endogenous IL-2 production.²⁸ When the frequency of T cells producing different cytokines simultaneously

was monitored, a substantial proportion of IFN- γ - and IL-2-producing cells expressing both cytokines together was found, whereas the frequency of IL-4+ IFN- γ double-positive cells was extremely low, indicating that these two cytokines are independently expressed. Our finding that atopic T cells expressing IL-2 and IFN- γ simultaneously on stimulation were identified in a lower frequency when compared with healthy T cells shows that a T_H1-like T-cell population is decreased in the peripheral blood of atopic patients.

Most of our patients with asthma were treated with inhaled beclomethasone. To determine whether this medication contributes to reduced IFN- γ production in atopic individuals, two healthy volunteers were treated with inhaled beclomethasone (600 μ g/day) for 7 days. Peripheral lymphocytes were obtained on 4 days, stimulated, and tested for IFN- γ production. No alteration of IFN- γ production was observed. This finding is in accordance with studies showing that inhalation of beclomethasone up to 800 μ g/day may not have

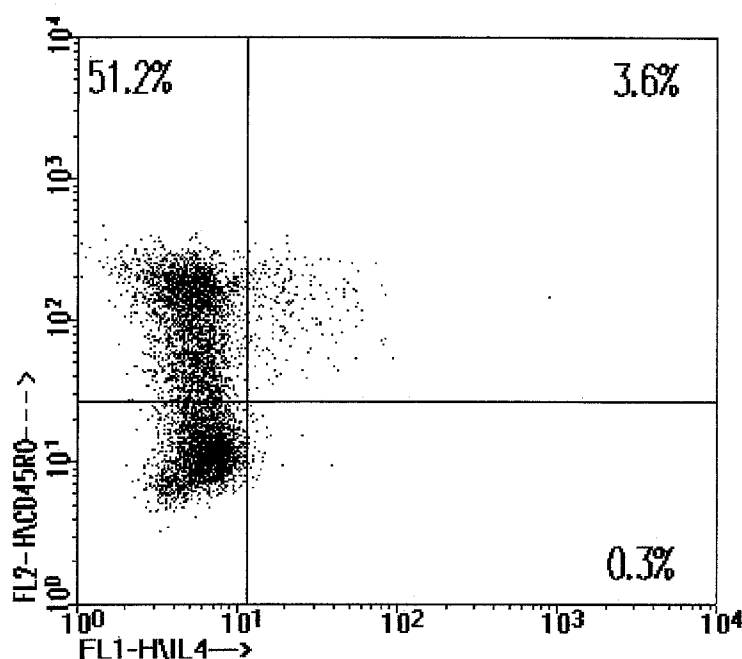


FIG. 3. PBMCs from a healthy donor were stimulated as described in the legend to Fig. 2. Cells were stained for IL-4 (horizontal), CD45R0 (vertical), and CD3 (third color, not shown). An analysis gate was set on lymphocytes by scatter properties and CD3+ cells. The dot plot shows that cells could be discriminated into CD45R0+ and CD45R0- T cells. Only cells expressing CD45R0 produced IL-4 (double-positive).

TABLE IV. IFN- γ and IL-4 production of stimulated CD3+ CD45R0+ (CD45RA-) and CD3+ CD45R0- (CD45RA+) cells

	IFN- γ (U/ml)	IL-4 (pg/ml)
AP CD3+CD45R0+	15.5 (9.4-26.6)	61.9 (<DL-353.2)
NAS CD3+CD45R0+	35.7 (16.8-59.0)	76.5 (30.2-1180)
AP CD3+CD45R0-	7.7 (2.2-17.4)	<DL
NAS CD3+CD45R0-	21.7 (6.3-36.8)	<DL

AP, Atopic patients; DL, detection limit; NAS, nonatopic subjects.

T-cell subsets were obtained as described in the Methods section and stimulated with PMA and ionomycin. Culture supernatants were harvested after 24 hours and analyzed for IFN- γ and IL-4. Patient group ($n = 5$), control group ($n = 5$); median and range are listed.

systemic effects,^{29,30} but higher doses (up to 2000 μ g/day) may.³¹ When peripheral lymphocytes were stimulated in vitro with PMA and ionomycin, salbutamol did not alter the frequency of IFN- γ -producing cells. Dexamethasone could decrease the number of IFN- γ -producing cells; however, high doses (up to 1000 ng/ml) were required. Thus

it seems unlikely, that medication could explain reduced IFN- γ production in atopic patients. However, high-dose steroid therapy may contribute to a reduced capability to produce IFN- γ . Recently, it was shown that 1-year-old infants with atopic symptoms had exhibited a decreased IFN- γ production when cord blood was examined at birth.³² Again, this observation argues against a major role of patients' medication and confirms the hypothesis of an intrinsic defect to produce IFN- γ in atopic diseases.

Interestingly, we could not find an enhanced frequency of peripheral T cells producing IL-4 in the atopic group, regardless of whether CD4+ or CD8+ T cells were analyzed and regardless of the stimulus. As demonstrated here and by others,^{14,16,17} IL-4-producing T cells were exclusively of the CD45R0+ memory phenotype. When the number of IL-4-producing cells was related to the cell surface marker CD45R0, a slight elevation was found in the atopic group compared with the healthy group, but it did not reach statistical significance. To corroborate these results, we quantitated IL-4 production in supernatants of CD45R0+ T cells, activated either with phorbol ester and ionomycin or PHA. In both cases, we could not observe enhanced IL-4 production. This is in contrast to other reports, which show that

culture supernatants of unfractionated PBMCs from atopic patients produced more IL-4 than those from healthy control subjects⁷⁻¹⁰ as determined by ELISA^{7,9} or bioassay.^{8,10} Although bioassays may have high sensitivities, the results of bioactivity are not necessarily comparable to immunoreactivity obtained with monoclonal antibodies in immunoassays. Thus monitoring of IL-4-induced CD23 expression on RAMOS cells may not correspond to ELISA results.^{8,10} The cited groups^{7,9} used activated PBMCs for IL-4 analysis by ELISA, whereas we performed our experiments in part with sorted CD45R0+ T cells to circumvent any T-cell subset imbalance among the donors. This approach may be a useful method for obtaining information about a defined cell population. On the other hand, an isolated CD45 restricted T-cell subset may have altered immune functions when the CD45 counterpart is missing, as has been recently demonstrated in vivo³³ and in vitro.³⁴ This would imply that naive T cells may help memory T cells to produce IL-4. We do not have evidence for such a hypothesized pathway, but we have recently shown that human memory T cells could provide help for naive T cells to produce IFN- γ as measured with our intracellular staining technique.³⁵ However, when IL-4 production of PBMCs was investigated, we could not find a difference between the atopic and control groups.

Although our results do not suggest a role for overproduction of IL-4 in atopic diseases, they do not argue against a role for IL-4 in the pathogenesis of these diseases. Peripheral blood contains only 2% of all lymphocytes and may not contain highly differentiated antigen-specific T_{H0}, T_{H1} or T_{H2} cells, which are localized in inflammatory tissues. Thus a dominant population of IL-4-producing T cells in inflamed skin or bronchial tissue has been observed in a number of studies^{2-4, 36-38} in which cloning procedures and in situ hybridization techniques were used. Because IL-4 was shown to be necessary for the development of IL-4 production,³⁹ it is possible that localized high IL-4 production in inflamed tissue may help switching of memory T cells to T_{H0} or T_{H2} cells.

Furthermore, enhanced IgE production in atopic patients might not simply be due to elevated IL-4 production. It was shown that naive CD45RA+ T cells were strong inducers of IL-4-driven IgE production, which could be blocked by the addition of IL-2, whereas CD45R0+ T cells needed IL-2 and IL-4 to exhibit their IgE helper

effect.⁴⁰ Furthermore, activation of CD45RA+ naive T cells could be achieved by anti-T-cell-receptor and IL-4 stimulation, suggesting an increased expression of functional IL-4 receptors on naive cells.⁴¹ Thus an imbalance in cells bearing different CD45 isoforms could also be responsible for a dysregulated IgE synthesis in atopic patients. IFN- γ is a potent inhibitor of IgE synthesis,⁴² and defective production may play a role in in vivo IgE synthesis. Evidence was provided by the demonstration that pokeweed mitogen, which usually does not induce IgE production, could stimulate IgE synthesis in vitro when IFN- γ signalling was inhibited by a blocking anti-IFN- γ receptor antibody.⁴³ The recently described cytokine IL-13 also supports IgE synthesis in vitro in the absence of IL-4,⁴⁴ indicating that other, still unknown, soluble mediators may be involved in the regulation of IgE production.

In conclusion, our findings at the single cell level, provide evidence for a defect in the ability of peripheral CD4+ T cells of atopic patients to produce IFN- γ . In contrast, no abnormality in the capacity for IL-4 production was identified. Additionally, all tested T-cell subsets from these patients had a reduced frequency of IL-2-producing cells suggesting a deficiency in producing T_{H1}-like cytokines. Our studies demonstrate the critical importance of measuring cytokine production in defined lymphocyte subpopulations. Such a strategy may help us to learn more about the role of dysregulated cytokine production in individual cells and its contribution to the pathogenesis of atopic diseases.

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