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Evidence that defective interferon-gamma production in atopic dermatitis patients is due to intrinsic abnormalities

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SUMMARY

The *in vitro* production of interferon-gamma (IFN-γ) in 19 atopic dermatitis (AD) patients was compared with that of 12 controls. IFN-γ production by phytohaemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMC) was profoundly diminished in AD patients, whereas the proliferative response was similar to that of control PBMC. The addition of 40 U/ml of interleukin-2 (IL-2) to the cultures failed to restore IFN-γ production. Similarly, removal of adherent cells also had no effect. Reduced IFN-γ secretion was observed after stimulation with the CD3 monoclonal antibody OKT3, ionomycin + 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or with high levels of IL-2 (200 U/ml). There were increased proportions of $CD4^+$ T helper/inducer cells and decreased proportions of $CD8^+$ T cytotoxic-/suppressor cells and $CD16^+$ natural killer (NK) cells in AD patients. This resulted in an increased CD4/CD8 ratio as compared with controls, but no correlation was observed between numbers of T cell subpopulations and IFN-γ generation. However, a significant correlation was found between IFN-γ generation *in vitro* and IgE serum concentration in AD patients. The data suggest that the decreased production of IFN-γ by AD patients is due to intrinsic differences in capacity to produce this cytokine and is not the result of differences in regulatory cell interactions. Moreover, the findings indicate that decreased production of IFN-γ may be an important factor in the pathogenesis of this disease.

Keywords atopic dermatitis interferon-gamma IgE

INTRODUCTION

Atopic dermatitis (AD) is a chronic, inflammatory skin disease with multiple immunologic abnormalities including defective regulation of IgE synthesis, decreased number of circulating $CD8^+$ T cells and $CD16^+$ natural killer (NK) cells, impaired NK cell activity, decreased suppressor cell function, impaired autologous mixed lymphocyte reactions, and depressed granulocyte and monocyte chemotaxis (Leung & Geha, 1986). Because of the multiplicity of immune defects in AD, abnormalities in regulatory T cell subsets and lymphokines might be involved.

It has been shown in the murine system that IgE synthesis is under the control of two T cell subsets (T_{H1} AND T_{H2}), which are defined according to the different lymphokines released (Mosmann & Coffman, 1987). Although a clear dichotomy between different lymphokine-producing T_H cells does not seem to exist in humans, recent data have shown that human IgE synthesis is regulated by different factors produced by activated T lymphocytes, including interleukin-4 (IL-4) and interferon-gamma (IFN-γ) (Snapper, Finkelman & Paul, 1988). IL-4 and IFN-γ have been shown to have reciprocal roles in the regulation of IgE responses *in vitro* and *in vivo*; IL-4 promotes and IFN-γ inhibits its production (Pene *et al.*, 1988). We have reported a profound defect of IFN-γ production *in vitro* after phytohaemagglutinin (PHA) stimulation of leucocytes from peripheral blood from patients with severe AD. This was negatively correlated with their *in vivo* IgE titre (Reinhold *et al.*, 1988). This has been confirmed by other investigators (Rousset *et al.*, 1988; Boguniewicz *et al.*, 1989). Defective IFN-γ generation suggests a major role in the pathogenesis of increased IgE production and increased expression of its receptor in these patients. Moreover, since IFN-γ has numerous effects on cells of the immune system, including anti-viral and growth inhibitory activities, activation of macrophages, activation and growth enhancement of cytolytic T lymphocytes and NK cells and the induction of class II major histocompatibility complex (MHC) antigen expression, defective production of this lymphokine may have serious immunologic consequences that may be related to pathological conditions seen in AD. Here we present a detailed study of the *in vitro* IFN-γ production in AD patients.

PATIENTS AND METHODS

Subjects

Peripheral blood mononuclear cells (PBMC) of 19 patients with

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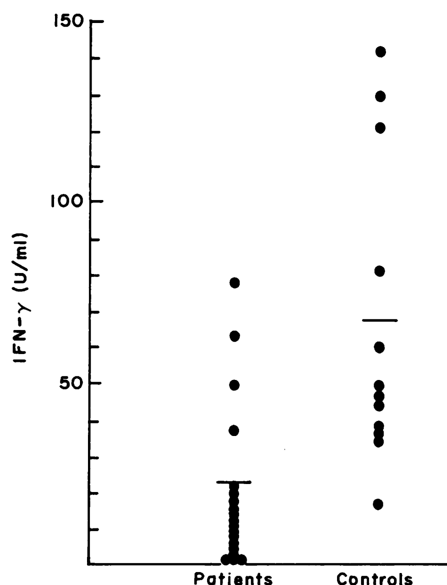

Fig. 1. Reduced interferon-gamma (IFN-γ) production in response to phytohaemagglutinin (PHA) in hyper-IgE atopic dermatitis patients ($P < 0.05$).

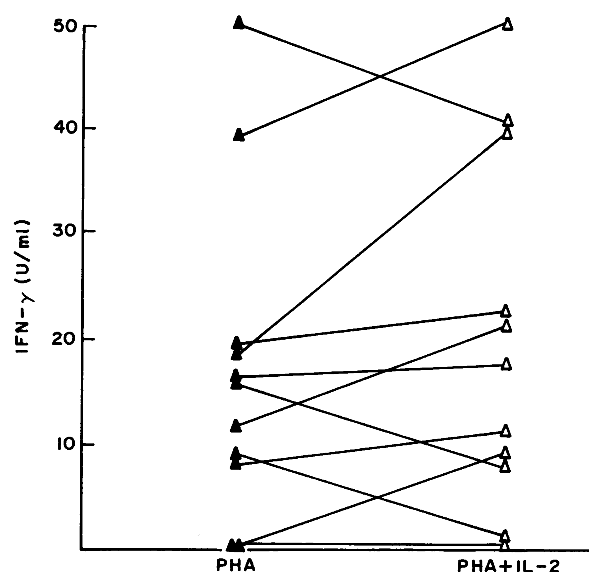

Fig. 2. Effect of interleukin-2 (IL-2) on phytohaemagglutinin (PHA) induced interferon-gamma (IFN-γ) production in hyper-IgE atopic dermatitis patients.

severe AD were studied (11 men and eight women, aged 26 ± 10 years). The patients were selected from the Department of Dermatology, University of Bonn. Diagnosis was established according to the criteria of Hanifin & Rajka (1980). All patients had the exacerbated severe form of AD involving more than 50% of the body area; 12 patients had a marked elevation (1000 to 25 000 U/ml IgE) and five patients a moderate elevation (300 to 1000 U/ml IgE) of their serum IgE concentration. Two patients showed a normal IgE serum concentration. All patients had severity score factor >7 i.e. severe disease as defined by Rajka (1983). None of the patients was receiving oral steroids at the time and not for 6 months beforehand. PBMC from 12 healthy subjects without symptoms and history of atopy were also studied (five women and seven men aged 27 ± 5); their serum IgE was < 100 IU/ml.

Cell separation and cultivation

Heparinized blood (50 IU/ml) was collected from different donors, diluted with phosphate-buffered saline (PBS) and PBMC were isolated using standard Ficoll–Isopaque gradient centrifugation. Cells were washed three times with PBS and finally suspended in RPMI 1640+20 mM HEPES+2 mM L-glutamine (Gibco, Karlsruhe, FRG), penicillin (100 IU/ml) + streptomycin (100 μg/ml; Seromed, Berlin, FRG) and 10% fetal calf serum (FCS) (Seromed). Cells were cultured in the presence or absence of an optimal stimulating concentration of PHA at a dose of 1% stock solution (unpurified, batch 13N1076; Gibco), 10 ng/ml 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Sigma, Munich, FRG) and 400 ng/ml ionomycin (Io; Calbiochem, San Diego, CA), 25 ng/ml anti-CD3 antibody (OKT3; Ortho Pharmaceutical, Heidelberg, FRG), and 200 U/ml purified natural IL-2 (Lymphocult T-HP, Biotest, Frankfurt, FRG). In addition, purified natural IL-2 at 40 U/ml was added to unstimulated and PHA-stimulated PBMC at the beginning of the cultures. In separate experiments adherent cells were removed by incubation of mononuclear cells on 60-mm plastic tissue culture dishes (Falcon, Becton Dickinson, Heidelberg, FRG) for 1 h at 37°C. Non-adherent cells were removed by washing with warm medium. Final suspensions contained $< 5\%$ CD14 (anti-LeuM3) positive monocytes as determined by FACS analysis. Cultures were distributed in 2-ml aliquots into 24-well Costar culture plates (Tecnomara, Fernwald, FRG) at 1×10^6/ml for a period of 24 h at 37°C, 5% CO_2. Supernatants were harvested after centrifugation and stored at −70°C until the time of the assay.

Table 1. *In vitro* interferon-gamma (IFN-γ) production by peripheral blood mononuclear cells of healthy donors and of patients with atopic dermatitis, induced by phytohaemagglutinin (PHA), OKT3, interleukin-2 (IL-2), and a combination of calcium ionophore (Io) with phorbol ester (TPA).

	IFN-γ(U/ml)			
	PHA	OKT3	IL 2	Io+TPA
Patients ($n=19$)	21±22*	7±7*	3±5	277±170*
Controls ($n=12$)	67±43	50±45	39±63	735±345

Stimulating agents were used at concentrations previously determined by titration to be optimal for stimulation (data not shown), as follows: PHA, 1%; OKT3, 25 ng/ml; IL-2, 200 U/ml; Io, 400 ng/ml; TPA, 10 ng/ml.
* $P < 0.005$

Proliferation assays

^{3}H-thymidine incorporation assays were performed in triplicate in 96-well round-bottomed microtitre plates (Greiner, Nür-

Table 2. Effect of removal of adherent cells on *in vitro* interferon-gamma (IFN-γ) production induced by phytohaemagglutinin (PHA)

	IFN-γ	(U/ml)*
	PBMC	Non-adherent cells
Control		
1	39	60
2	92	172
3	58	72
4	46	50
Mean ± s.d.	59 ± 24	89 ± 56
Patient		
1	8	0
2	8	12
3	12	16
4	0	0
5	22	13
Mean ± s.d.	10 ± 8	8 ± 8

* IFN-γ in U/ml released by the addition of 1% PHA to peripheral blood mononuclear cells (PBMC) or to non-adherent cells after 24 h. Adherent cells were removed by plastic adherence.

tingen, FRG) in culture medium consisting of RPMI 1640 with 20 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS. PBMC were cultured at a concentration of 1×10^5/well for 96 h at 37°C (5% CO_2) with or without PHA at a final concentration of 1%. This concentration of PHA was found to be optimal when tested on PBMC from healthy donors (data not shown). ^{3}H- thymidine (37 kBq/well; Du Pont, Dreieich, FRG), was added during the last 18 h of culture. Incorporated radioactivity was quantified using a liquid scintillation counter (Beckmann Instruments, Munich, FRG).

IFN-γ assay

The amount of IFN-γ released into the supernatants was measured by an ELISA (Holland Biotechnology, Leiden, The Netherlands) which uses monoclonal antibodies (MoAbs) to detect IFN-γ. OD values were converted into units using the values of a standard IFN-γ dilution curve. The assay was sensitive to a lower limit of 3 U/ml IFN-γ.

Serum IgE quantification

Serum IgE concentrations were determined by a solid-phase radioimmunoassay (Phadebas IgE Prist; Pharmacia, Freiburg, FRG).

Expression of surface antigens

Ficoll–Isopaque-purified PBMC were stained with fluorescein isothiocyanate (FITC) conjugated MoAbs to CD3 (anti-Leu-4), CD4 (anti-Leu-3a), CD8 (anti-Leu-2a), CD16 (anti-Leu-11a) (all from Becton Dickinson). PBMC (5×10^5) were incubated on ice for 30 min with 1 μg/ml of MoAb in PBS containing 2% FCS and 0·1% sodium azide. After two washes the number of fluorescent cells was determined on a FACS analyser (Becton Dickinson). The absolute number of fluorescent lymphocytes was calculated from total white blood cell count determined on a Coulter Counter (Coulter Electronics) and a 200-cell manual differential blood count.

Statistical analysis

The differences between data sets were assessed using the Mann–Whitney *U*-test. Data are expressed as mean ± s.d. For correlation analysis the Spearman correlation coefficient was used.

RESULTS

IFN-γ production in response to PHA

Nineteen AD patients and 12 controls were tested for their IFN-γ production *in vitro*. There was no spontaneous IFN-γ detected in the PBMC supernatants from any of the groups tested. In the presence of 1% PHA, IFN-γ production reached a maximum at 24 h, remained unchanged up to 48 h and decreased thereafter to low levels (data not shown). As shown in Fig. 1, PHA-induced IFN-γ production (at 24 h) in patients with AD (mean 21 ± 22 U/ml) was significantly lower ($P < 0{\cdot}005$) compared with controls (mean 67 ± 43 U/ml). This did not reflect a difference in the kinetics of IFN-γ production in healthy subjects and patients with AD, since both showed maximal IFN-γ release at 24 h (not shown). In contrast to the decreased production of IFN-γ by PBMC, replication of these cells in response to PHA, as indicated by ^{3}H-thymidine incorporation, was similar to that by control PBMC (patients, 142919 ± 47270; controls, 119635 ± 43102).

Effect of IL-2 addition on PHA-induced IFN-γ production

We next investigated the ability of IL-2 to restore IFN-γ production in AD patients. When double stimulation, with PHA and IL-2 (40 U/ml), was employed, the response was essentially similar to that with PHA alone (Fig. 2). IL-2 supplementation did not significantly increase production of IFN-γ by PBMC in AD patients and controls. IL-2 alone (40 U/ml) did not induce IFN-γ production in resting unstimulated PBMC from either patients or controls in these experiments. At higher concentrations IL-2 can induce IFN-γ production in the absence of other stimuli. A concentration of 200 U/ml of IL-2 induced significant amounts of IFN- in control PBMC whereas IFN- production in AD patients was severely depressed (Table 1).

Induction of IFN-γ by anti-CD3 or the combination of calcium ionophore (Io) with TPA

As shown in Table 1, PBMC of AD patients stimulated with OKT3 produced very low titres of IFN-γ (mean 7 ± 7 U/ml) compared with controls (mean 50 ± 45 U/ml). Io + TPA induced IFN-γ production in both groups but PBMC from AD patients produced significantly less IFN-γ than did PBMC from control individuals (Table 1).

Effect on removal of adherent cells on PHA-induced IFN-γ production

PBMC from patients and from controls were depleted of monocytes by plastic adherence in order to investigate their possible suppressive role in the production of IFN-γ. IFN-γ production remained unchanged in PBMC from five patients

Table 3. Phenotypic profile of T lymphocyte subpopulations

	Positive cells		Absolute Number Positive cells†			
Study group	Leu-4 (CD3)	Leu-3 (CD4)	Leu-2 (CD8)	Leu-11 (CD16)	T subset ratio ($CD4^+$:$CD8^+$)	Absolute lymphocyte numbers (mean positive cells/mm^3)
AD patients	74±5 (1717±766)	55±8‡ (1308±695)	15±7‡ (313±163)‡	4±3* (96±64)‡	5·0±3·2‡	2320±1047
Controls	71±5 (1456±317)	46±9 (929±303)	23±6 (472±137)	7±3 (156±86)	2·2±0·8	2028±358

* Percentage of positive cells within the entire peripheral lymphocyte population, determined by flow cytometry; mean ± s.d.
† Mean number of positive cells/mm^3 peripheral blood ± s.d.
‡ $P<0{\cdot}05$
AD, atopic dermatitis.

Table 4. Corelation analysis between *in vitro* interferon-gamma (IFN-γ) production and other biological parameters in patients with atopic dermatitis

	CD3	CD4	CD8	CD16	T subset ratio	Absolute lymphocyte number	log IgE
r	0·31	−0·23	0·12	−0·24	−0·17	−0·35	−0·68
P	0·13	0·20	0·34	0·22	0·27	0·09	0·004

with AD but was clearly enhanced in three out of four control subjects (Table 2).

Phenotypic profile of T cell subsets
Patients with AD showed a significant depletion of T cells in the Leu-2 (CD8) subset and a significant increase of the percentage of Leu-3 (CD4) positive cells compared with controls (Table 3). These differences are reflected in the ratios of T helper/inducer cells to T suppressor/cytotoxic cells (Leu-3/Leu-2), which was significantly increased in AD patients compared with controls. AD patients showed significantly decreased numbers of total $CD16^+$ NK cells.

Correlation analysis between IFN-γ production and other biological parameters
PHA-induced IFN-γ generation *in vitro* and IgE serum concentration in patients with AD was strongly negatively correlated ($r=-0{\cdot}68$; $P<0{\cdot}005$), whereas no significant correlation was observed with $CD3^+$, $CD4^+$ or $CD8^+$ lymphocytes, $CD4^+$/$CD8^+$ ratios, $CD16^+$ NK cells or absolute lymphocyte numbers (Table 4).

DISCUSSION

In this study we performed a series of experiments to examine mechanisms for the decreased production of IFN-γ in a subgroup of patients with AD. Investigating a larger group of AD patients with elevated IgE serum levels we have confirmed previous data on severe deficiency of IFN-γ synthesis using PHA as the stimulating agent (Reinhold *et al.*, 1988). Recently, Bonguniewicz *et al.* (1989) observed defective IFN-γ production of PBMC from AD patients after stimulation with another lectin, concanavalin A (Con A). Since earlier studies concluded that IFN-γ inducing activity of mitogens could be mediated by IL-2 release from activated T cells (Vilcek *et al.*, 1985), we examined whether addition of IL-2 to PHA-induced PBMC could restore defective IFN-γ production. *In vitro* addition of 40 U/ml of IL-2 could not restore effectively IFN-γ in patients examined in this study. These findings suggest that defective IL-2 production was not the cause of decreased IFN-γ production. Higher concentrations of IL-2 known to induce IFN-γ synthesis in the absence of mitogens also resulted in defective IFN-γ production in AD patients compared with controls. The cells that produce IFN-γ upon stimulation by IL-2 have been identified as T cells and especially NK cells (Kasahara *et al.*, 1983; Young & Ortaldo, 1987). In contrast to the decreased production of IFN-γ by PBMC, proliferation in response to PHA was similar to that by control PBMC. However, previous studies have shown that production of IFN-γ and cellular proliferation are not obligatorily coupled events (Johnson, Farrar & Torres, 1982).

Since defective IFN-γ production may be due to abnormal regulation of T lymphocyte functions, another possibility is that it can be the result of a suppressive mechanism such as suppressive monocytes. Monocytes have been found to be responsible, at least in part, for several deficient *in vitro* T and B lymphocyte responses of PBMC (Tilden & Balch, 1982; Combe *et al.*, 1984). However, in this study monocytes were excluded as a source of suppression of IFN-γ production in AD patients. Other cellular sources of suppression were not investigated.

We show that the defect can also be observed after stimulation with a MoAb for T lymphocytes (OKT3). The OKT3 MoAb, specific for the CD3 complex, induces IFN-γ synthesis in cells of the T lymphocyte lineage (Chang *et al.*, 1982), which suggests that T lymphocytes in PBMC of AD patients are defective in production of IFN-γ. In the case of PHA or OKT3 induction, an interaction between T cells and accessory cells is necessary for IFN-γ production. This is related to the antigen-presenting capacity, as well as possibly IL-1 production by accessory cells. Since it has been shown that monocyte function is defective in AD patients (Raesaenen *et al.*,

1987), we tested IFN-γ production in AD patients using the combination of calcium ionophore (Io) and phorbolester (TPA) as stimulating agents which circumvent the requirement of accessory cells and IL-1 (Truneh *et al.*, 1985). Activation by Io+TPA induced significant amounts of IFN-γ in hyper IgE AD patients but the response was still markedly less than that by controls. These data suggest that decreased IFN-γ production by AD patients may be intrinsically limited. Gauchat *et al.* (1988) have recently found that decreased IFN-γ production by atopic cells appears to be due to a pretranslational deficiency, since Northern blots of RNA from PWM-stimulated PBMC that were hybridized to an IFN-γ probe revealed bands that were much less intensive as with RNA from comparable adult cells. This could be secondary to either failure of transmission of the signal to the nucleus or to primary transcriptional abnormality. Io and TPA are known to activate protein kinase C and to raise cytosolic free Ca^{2+} concentration resulting in strong synergism in inducing the gene for IFN-γ (Hardy *et al.*, 1987).

Phenotypic analysis of PBMC confirmed preliminary findings of decreased numbers of $CD8^+$ T cells, which was reflected in the ratio of T helper/inducer to T suppressor/cytotoxic cells and was significantly increased in AD patients (Faure *et al.*, 1982). Moreover, AD patients showed significantly decreased numbers of $CD16^+$ NK cells which is consistent with previous findings (Reinhold *et al.*, 1986). In the present study IFN-γ production *in vitro* did not correlate with abnormalities in the distribution of T lymphocyte subpopulations and NK cells. Simple absence of IFN-γ producing lymphocyte subsets could be excluded since phenotypic analysis revealed increased rather than reduced proportions of $CD4^+$ T cells known as the primary source of IFN-γ in PBMC (Chang *et al.*, 1982). However, IFN-γ production was strongly negatively correlated with the serum IgE concentration *in vivo*, which is consistent with our previous data (Reinhold *et al.*, 1988). Since IFN-γ acts as a suppressive factor for IL-4-mediated IgE responses it seems possible that reduced IFN-γ production may account for enhanced levels of IgE observed in these patients. This is confirmed by the recent finding that T cells of atopic hyper-IgE patients produce enhanced levels of IL-4 (Rousset *et al.*, 1988). Furthermore, defective secretion of IFN-γ is likely to have several other consequences in terms of immunologic regulation. Immunologic abnormalities in AD are a low natural killer activity (Jensen *et al.*, 1984; Hall, Rycroft & Brostoff, 1985) and an absent expression of class II HLA antigens on keratinocytes (Barker, Ophir & MacDonald, 1987). Interestingly, the low NK activity can be increased by IFN-γ in these patients (Jensen *et al.*, 1984). These observations suggest that the defective NK activity and HLA-DR expression are consequences rather than cause of the IFN-γ defect. In a recently performed pilot study clinical improvement and decrease of spontaneous IgE production *in vitro* has been shown during *in vivo* treatment of AD patients with recombinant IFN-γ (Boguniewicz *et al.*, 1989). Thus, decreased production of IFN-γ may be an important factor in the pathogenesis of this disease. It is critical to identify the signals influencing IFN-γ production better, which could result in a better understanding of the defect in regulation of IFN-γ secretion observed in AD patients.

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