

Interferon- γ promotes exaggerated cytokine production in keratinocytes cultured from patients with atopic dermatitis

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Recent studies suggest that skin keratinocytes from patients with atopic dermatitis (AD) and nonatopic subjects differ in their intrinsic ability to respond to proinflammatory stimuli. In this study keratinocyte cultures established from the normal-looking skin of six adult patients with AD and six healthy, nonatopic control subjects were compared in their response to interferon (IFN)- γ , a potent proinflammatory lymphokine whose expression is increased in chronic AD lesions. Basal expression of IFN- γ receptor as well as IFN- γ -induced membrane expression of HLA-DR and intercellular adhesion molecule (ICAM)-1 were evaluated by flow cytometry. Keratinocyte release of IL-1 α , IL-1 receptor antagonist (IL-1ra), granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)- α were measured by ELISA on culture supernatants after treatment with IFN- γ or medium alone. Expression of membrane IFN- γ receptor was similar in keratinocytes cultured from nonatopic subjects and subjects with AD. IFN- γ (10 to 500 U/ml) induced comparable levels of membrane HLA-DR and ICAM-1 in both groups of keratinocytes. In contrast, spontaneous release of IL-1 α , IL-1ra, GM-CSF, and TNF- α was increased in the supernatants of unstimulated keratinocytes from patients with AD compared with keratinocytes from control subjects, with IL-1ra and GM-CSF reaching statistically significant difference. Moreover, IFN- γ -induced release of all the cytokines tested was much higher for keratinocytes from patients with AD, but the IL-1ra/IL-1 α ratio for the two groups of keratinocytes was not substantially different, either basally or after IFN- γ stimulation. The results indicate that keratinocytes from patients with AD are hyperresponsive to IFN- γ in terms of cytokine release. (*J Allergy Clin Immunol* 1998;101:538-44.)

Key words: Atopic dermatitis, keratinocytes, cytokines, adhesion molecules, interferon- γ

Atopic dermatitis (AD) is associated with an abnormal activation and expansion of distinct subpopulations of helper T lymphocytes (T_H) both in the peripheral blood and in the skin. In particular, initiation and maintenance of AD are believed to be caused by infiltration of T_{H2} cells,¹⁻⁵ because the lymphocyte-derived

Abbreviations used

AD:	Atopic dermatitis
FITC:	Fluorescein isothiocyanate
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
ICAM-1:	Intercellular adhesion molecule-1
IFN:	Interferon
IL-1ra:	IL-1 receptor antagonist
mAb:	Monoclonal antibody
T _H :	Helper T lymphocytes
TNF- α :	Tumor necrosis factor- α

cytokines predominantly found in skin lesions are IL-4, IL-5, and IL-10, with lower amounts of interferon (IFN)- γ .⁶⁻⁸ A reduced IFN- γ production has also been correlated with the increased tendency to develop skin infections and, together with a higher synthesis of IL-4, IL-5, and their respective receptors, is considered responsible for enhanced IgE synthesis.⁹ The observations that T_{H2} cytokines could be determinant factors in the pathogenesis of AD prompted the introduction of IFN- γ in the treatment of severe cases of AD, with the intent to counteract dysregulated cytokine production and inhibit T_{H2}-mediated immune responses.¹⁰⁻¹² Interestingly, some authors reported that serum levels of the proinflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) increased during the first month of IFN- γ administration.¹³

Resident skin cells can provide a strong contribution to the expression of inflammatory skin disorders. In particular, keratinocytes can produce chemokines that attract inflammatory cells and cytokines that affect leukocyte functions. They can also express membrane molecules important for retention and activation of T cells.¹⁴ IFN- γ is a well-characterized, potent proinflammatory stimulus in a variety of biologic systems.¹⁵ Human keratinocytes express high-affinity receptor sites for IFN- γ ,¹⁶ and IFN- γ -activated keratinocytes synthesize major histocompatibility class II molecules¹⁷ and adhesion molecules, such as intercellular adhesion molecule (ICAM)-1.¹⁸ In addition, IFN- γ is a potent inducer of cytokine and chemokine synthesis in keratinocytes.^{19, 20} Intradermal injection of IFN- γ in humans elicits a transient skin inflammation, with T-cell and monocyte infiltration and dermal and epidermal HLA-DR and ICAM-1 expression.²¹ Finally, a transgenic mouse with

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Supported by grants from the Italian Ministry of Health.

Received for publication Oct 1, 1997; revised Dec. 22, 1997; accepted for publication Dec. 22, 1997.

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0091-6749/98 \$5.00 + 0 1/1/88663

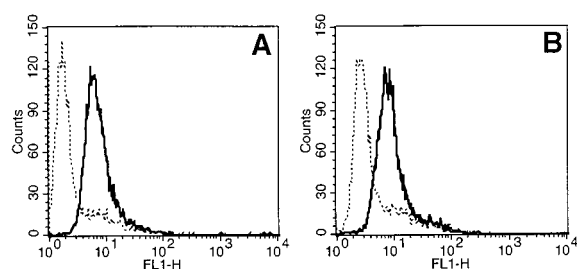


FIG. 1. Keratinocytes cultured from healthy, nonatopic subject (A) and patient with AD (B) express similar levels of IFN- γ receptor. Results are representative of experiments performed on keratinocyte cultures established from six nonatopic control subjects and six patients with AD. Dotted lines represent staining with isotype-matched control antibody.

stable expression of IFN- γ in the epidermis has provided an interesting model to study long-term effects of this cytokine on the skin, showing a series of skin changes strongly resembling eczema.²² Recently, keratinocytes from patients with AD have been reported to overproduce GM-CSF, a feature that can explain the high number and enhanced immunostimulatory functions of dendritic antigen-presenting cells in AD lesions,²³ suggesting that keratinocytes of patients with AD synthesize exaggerated amounts of certain cytokines in response to proinflammatory stimuli.

In this study we compared some of the proinflammatory effects of IFN- γ on keratinocyte cultures established from normal skin of adult patients with AD and healthy control subjects. Membrane expression of HLA-DR and ICAM-1, as well as the release of IL-1 α , IL-1 receptor antagonist (IL-1ra), GM-CSF, and tumor necrosis factor (TNF)- α , were measured in keratinocytes cultured from patients with AD after treatment with IFN- γ or medium alone. Keratinocytes cultured from healthy, nonatopic individuals served as controls.

METHODS

Subjects

Six adult patients with moderate-to-severe chronic AD and six healthy control subjects were included in the study. Patients received a definite diagnosis of AD according to standard criteria.²⁴ They were not receiving any systemic therapy for at least 2 weeks before testing, and the only medications allowed were limited amounts of topical corticosteroids. Two patients (women, 16 and 31 years of age) had only skin lesions, whereas four patients (two women, 15 and 26 years of age, and two men, 29 and 38 years of age) had also mucosal manifestations (rhinoconjunctivitis, asthma, or both). Serum IgE level was over 2000 kU/ml in one case, and between 104 and 671 kU/ml in the other five cases. Control subjects (three women and three men, age range 18 to 42 years) had no personal or family history of AD, asthma, or hay fever, and serum IgE levels were within normal limits. The study was approved by the Istituto Dermatologico dell'Immacolata Ethical Committee.

Cytokines and antibodies

Recombinant human IFN- γ and mouse anti-human IFN- γ -receptor (IgG1) monoclonal antibodies (mAbs) were pur-

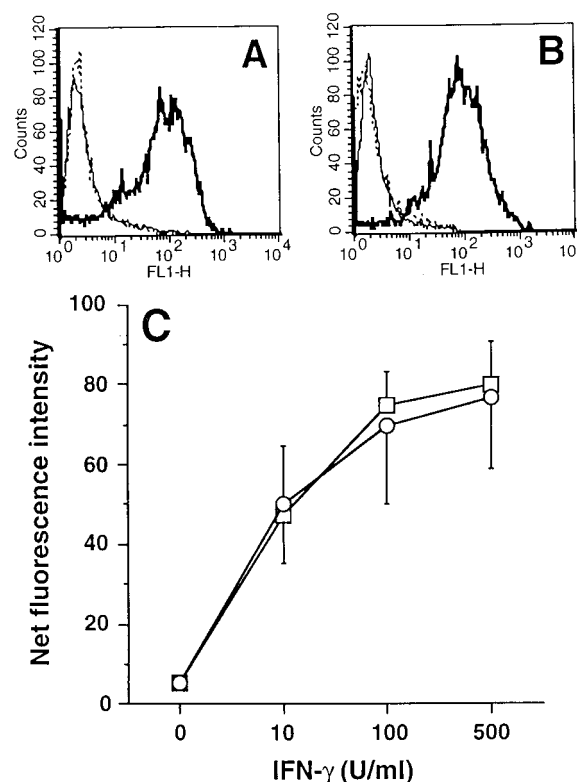


FIG. 2. Keratinocytes cultured from control individuals and patients with AD show similar induction of membrane HLA-DR after treatment with IFN- γ . Representative flow cytometry analysis of keratinocytes cultured from control subject (A) and patient with AD (B) stained for membrane HLA-DR after 48 hours of treatment with 100 U/ml IFN- γ . Dotted lines, staining with isotype-matched control antibody; continuous thin lines, staining of mock-treated keratinocytes with FITC-conjugated anti-HLA-DR mAb; and continuous bold lines, staining of IFN- γ -treated keratinocytes with FITC-conjugated anti-HLA-DR mAb. In C dose-response effect of IFN- γ (48 hours) on expression of HLA-DR in keratinocytes from nonatopic subjects (open circles) and subjects with AD (open squares) is shown. Numbers indicate mean fluorescence intensities of positive stainings subtracted from fluorescence of control antibody, and they are expressed as mean $\pm$ SD of data collected from staining keratinocytes cultured from six control subjects and six patients with AD.

chased from Genzyme Corporation (Cambridge, Mass.). Fluorescence isothiocyanate (FITC)-conjugated anti-HLA-DR (IgG2a) mAb was obtained from Becton-Dickinson (San Jose, Calif.), and FITC-conjugated anti-CD54 (ICAM-1, IgG1) was obtained from Immunotech (Marseille Cedex, France). FITC-conjugated anti-mouse immunoglobulin F(ab')₂ was purchased from Silenus (Hawthorn, Australia). Control mouse IgG1, and FITC-conjugated IgG1 and IgG2 were obtained from Immuno-

Keratinocyte cultures

Epidermal sheets for keratinocyte cultures were obtained from the roof of suction blisters raised on normal-appearing skin of the forearms of patients with AD. Epidermis of control subjects was prepared from suction-blisters roofs or from plastic surgery skin after separation of the epidermis from the dermis with 0.5% dispase (Boehringer Mannheim). To obtain single

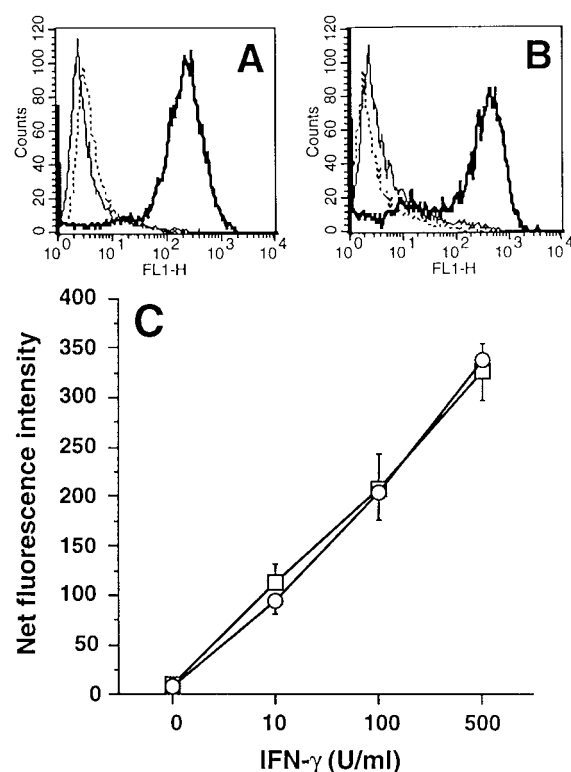


FIG. 3. Keratinocytes cultured from control subjects and patients with AD show similar induction of membrane ICAM-1 after treatment with IFN- γ . Representative flow cytometry analysis of keratinocytes cultured from control subject (**A**) and patient with AD (**B**) stained for membrane ICAM-1 after 48 hours of treatment with 100 U/ml IFN- γ . Dotted lines, staining with isotype-matched control antibody; continuous thin lines, staining of mock-treated keratinocytes with FITC-conjugated anti-ICAM-1 mAb; and continuous bold lines, staining of IFN- γ -treated keratinocytes with FITC-conjugated anti-ICAM-1 mAb. In **C** dose-response effect of IFN- γ (48 hours) on expression of ICAM-1 in keratinocytes from nonatopic subjects (open circles) and subjects with AD (open squares) is shown. Numbers indicate mean fluorescence intensities of positive stainings subtracted from fluorescence of control antibody, and they are expressed as mean $\pm$ SD of data collected from staining keratinocytes cultured from six control subjects and six patients with AD.

epidermal cell suspensions, epidermal sheets were rinsed in phosphate-buffered saline and then exposed to 0.25% trypsin (Biochrom KG, Berlin, Germany) for 30 minutes, followed by 5 minutes of vigorous pipetting. After trypsin neutralization with equal volume of medium with 10% fetal bovine serum (Delta Bioproducts, Johannesburg, South Africa), stratum corneum debris were removed with a 100 mm Falcon strainer (Becton Dickinson, Franklin Lakes, N.J.). Isolated epidermal cells were then seeded (1.2 to 2×10^4 /cm²) on a feeder layer of irradiated 3T3/J2 fibroblasts (2×10^4 /cm²) and cultured according to an optimized Rheinwald and Green culture technique, as described previously.²³ Contamination of keratinocyte cultures by leukocytes was excluded by flow cytometry analysis with mAbs against leukocyte membrane markers (not shown).

Flow cytometry analysis

IFN- γ -receptor expression was determined on second- or third-passage keratinocytes by using anti-IFN- γ -receptor mAb

followed by FITC-conjugated anti-mouse immunoglobulin. IFN- γ -induced HLA-DR and ICAM-1 expression was evaluated in keratinocytes cultured in 6-well plates and treated with IFN- γ (10 to 500 U/ml) or medium alone. After 24 to 72 hours, aliquots of 5×10^5 keratinocytes were stained for 30 minutes on ice with FITC-conjugated anti-HLA-DR and anti-ICAM-1 mAbs. In control samples, staining was performed with isotype-matched control antibodies or by omitting primary mAbs. The fluorescence was evaluated with a FACScan equipped with Cell Quest software (Becton Dickinson, Mountain View, Calif.).

ELISA

Second- or third-passage keratinocytes were cultured in 1.9 cm² wells at 1.5×10^3 cells/cm². During the assays, Rheinwald medium was used in the absence of hydrocortisone, containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) instead of 10% fetal bovine serum. Subconfluent keratinocyte cultures were treated with IFN- γ (10 to 500 U/ml) or medium alone. After 24 to 72 hours, supernatants were collected, filtered, and stored at -80° C, and keratinocytes were immediately detached and counted with a hemocytometer. They were greater than 90% viable, as estimated by the Trypan blue exclusion test. Cytokines were measured in the supernatants by using GM-CSF Predicta kit (Genzyme) and IL-1 α , IL-1 β , and TNF- α Quantikine kits (R&D Systems, Minneapolis, Minn.), according to the manufacturer's instructions. An ELISA reader (model 3550 UV; Biorad, Hercules, Calif.) was used. Keratinocyte cultures were carried out in triplicate for each condition and data are represented as picograms or nanograms per 10^6 cells.

Statistical analysis

The Mann-Whitney rank sum test was used to compare data of cytokine release from keratinocyte cultures of atopic and nonatopic subjects. Wilcoxon's signed rank test was applied for within-group comparisons before and after IFN- γ stimulation. Data were expressed as mean $\pm$ SD because they were normally distributed at the statistical elaboration (SigmaStat; Jandel Co., San Rafael, Calif.). Probability (p) values of 0.05 or less were considered significant.

RESULTS

Expression of IFN- γ receptor by keratinocytes

IFN- γ is a potent activator of many proinflammatory functions of keratinocytes. In addition, IFN- γ receptor has been indicated as one of the candidate genes involved in the predisposition to atopic disorders.²⁵ Therefore we first compared the expression of IFN- γ receptor on keratinocytes cultured from patients with AD and control subjects by flow cytometry analysis. As shown in Fig. 1, keratinocytes cultured from patients with AD and healthy, nonatopic subjects expressed similar amounts of membrane IFN- γ receptor.

IFN- γ -induced expression of HLA-DR and ICAM-1

IFN- γ is the most potent inducer of HLA-DR and ICAM-1 on keratinocytes.^{17, 18, 26} Incubation of keratinocyte cultures with different doses of IFN- γ for 24 to 72 hours promoted the expression of HLA-DR (Fig. 2) and ICAM-1 (Fig. 3), which was quantitatively similar in keratinocytes from patients with AD and nonatopic control subjects.

Release of IL-1 α , IL-1ra, GM-CSF, and TNF- α

In the next set of experiments, we compared cultured keratinocytes from patients with AD and control subjects for basal and IFN- γ -stimulated release of cytokines with an established important role in skin cell-mediated immune reactions. Spontaneous IL-1 α release at 48 hours was higher in subconfluent keratinocyte cultures from patients with AD compared with keratinocytes from control subjects (105.3 ± 110.7 vs 15.9 ± 7.42 pg/10⁶ cells, respectively), even though the difference was not statistically significant ($p = 0.093$). After 48 hours of stimulation with 100 U/ml IFN- γ , a dramatic increase in IL-1 α release was measured in both keratinocyte types, with keratinocytes prepared from patients with AD releasing much higher levels of IL-1 α than those from control subjects (1239.7 ± 645.7 vs 299.4 ± 156.15 pg/10⁶ cells, respectively; $p = 0.008$) (Fig. 4, A). Keratinocytes are strong producers of the specific antagonist to IL-1 receptors (IL-1ra), which is physiologically synthesized in large excess compared with IL-1 α .²⁷ Therefore we measured also IL-1ra content in the same supernatants (Fig. 4, B). In our experimental conditions we could detect a 10- to 100-fold excess of IL-1ra versus IL-1 α in unstimulated keratinocytes from control subjects and patients with AD. Compared with control subjects, significantly higher levels of IL-1ra were detected in the supernatant of keratinocytes from patients with AD, both basally (24.2 ± 26.4 vs 5.98 ± 1.36 ng/10⁶ cells; $p = 0.026$) and after 100 U/ml IFN- γ stimulation (116.9 ± 77.9 vs 26.80 ± 13.79 ; $p = 0.002$). On the other hand, calculation of the ratios between IL-1ra and IL-1 α concentrations in the same supernatants showed no significant differences in their values between keratinocytes from nonatopic subjects and patients with AD, both in untreated (391.6 ± 256.2 vs 309.9 ± 233.9) and in 100 U/ml IFN- γ -treated cultures (92.1 ± 39.0 vs 96.5 ± 48.3). Of note, IL-1ra/IL-1 α decreased markedly after IFN- γ stimulation because IFN- γ promoted a higher release of IL-1 α compared with IL-1ra (Fig. 4, C).

Because we had observed that keratinocytes from patients with AD produced enhanced amounts of GM-CSF, both spontaneously and after activation with phorbol esters or IL-1 α ,²³ we wanted to see whether IFN- γ was also effective in inducing GM-CSF release from keratinocytes. As shown in Fig. 5, A, keratinocytes derived from patients with AD released significantly higher levels of GM-CSF when compared with keratinocytes prepared from nonatopic control subjects, both spontaneously (222.5 ± 139.5 vs 8.2 ± 7.3 pg/10⁶ cells, respectively; $p = 0.002$) and after 100 U/ml IFN- γ treatment (1068.3 ± 575.3 vs 74.1 ± 108.3 , respectively; $p = 0.002$). Keratinocytes were also investigated for the release of TNF- α , which is primarily involved in skin inflammation by promoting adhesion molecule expression and cytokine release by resident and infiltrating cells¹⁴ (Fig. 5, B). Keratinocytes from patients with AD produced higher amounts of TNF- α , both spontaneously (17.0 ± 27.2 vs 0.09 ± 0.15 pg/10⁶ cells, respectively; $p = 0.13$) and after 100 U/ml IFN- γ stimulation (264.1 ± 185.4 vs 19.18 ± 11.21 , respectively; $p = 0.002$). Finally,

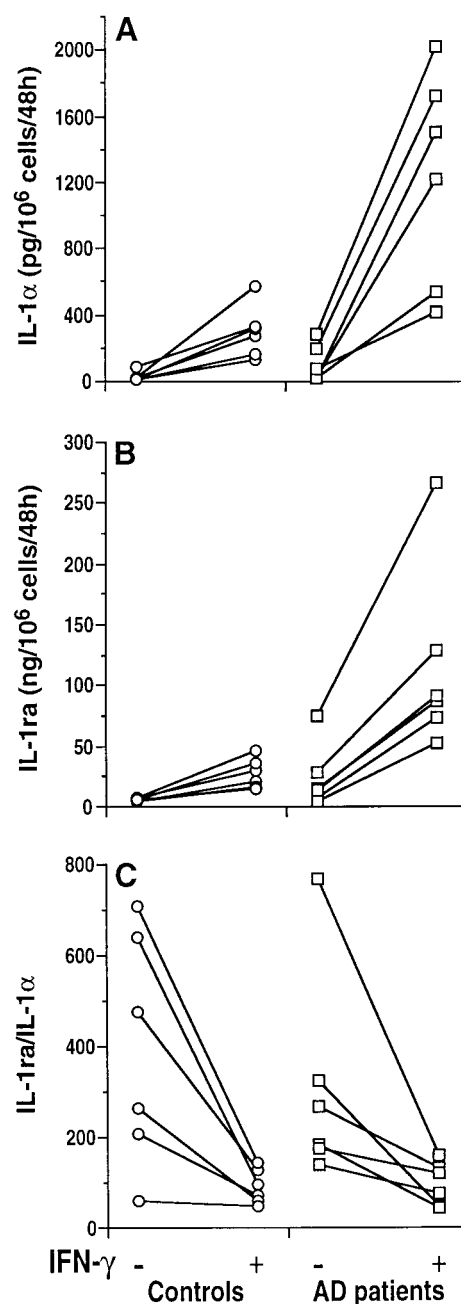


FIG. 4. Spontaneous and IFN- γ -induced release of IL-1 α (A) and IL-1ra (B) is enhanced in keratinocytes cultured from patients with AD (open squares) compared with keratinocytes from nonatopic subjects (open circles). IL-1ra/IL-1 α ratios diminish ($p < 0.01$) to similar extent in both keratinocyte groups after IFN- γ treatment (C). Release of IL-1 α and IL-1ra increase markedly on incubation with IFN- γ ($p < 0.01$) in both keratinocyte groups. Spontaneous release of IL-1 α and IL-1ra is higher in keratinocytes from patients with AD than in keratinocytes from nonatopic controls, but difference is statistically significant only for IL-1ra ($p < 0.03$). IFN- γ -stimulated release of IL-1 α and IL-1ra is strikingly higher in keratinocytes from patients with AD compared with keratinocytes from control subjects (IL-1 α , $p < 0.008$; IL-1ra, $p < 0.03$). Cytokine quantitation was performed by ELISA on supernatants collected after 48 hours of treatment with medium alone or in presence of 100 U/ml IFN- γ of subconfluent third-passage keratinocyte cultures from six nonatopic subjects and six patients with AD. Cultures were carried out in triplicate for each condition.

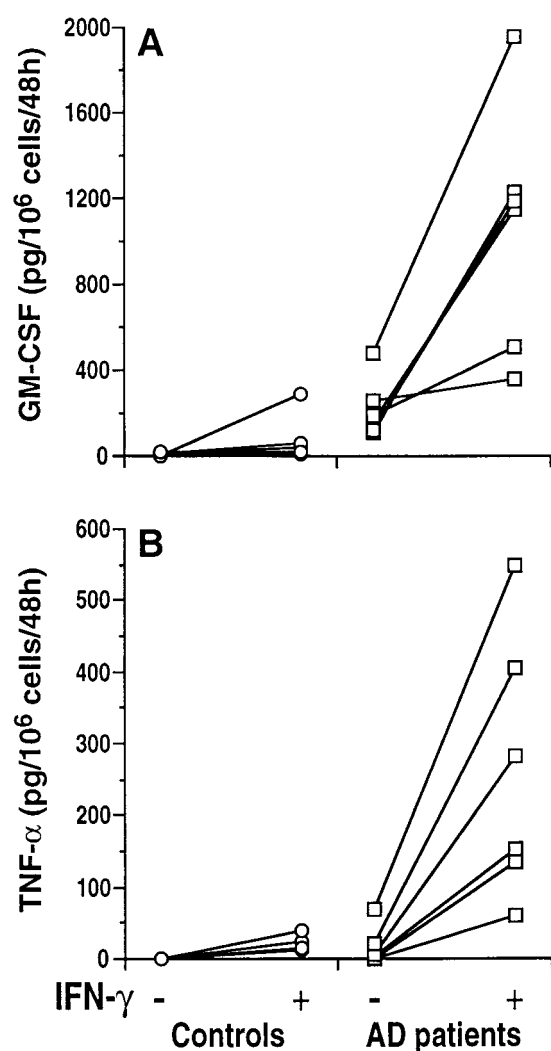


FIG. 5. Basal and IFN- γ -induced release of GM-CSF (**A**) and TNF- α (**B**) is increased in keratinocyte cultures from patients with AD (open squares) compared with keratinocytes from nonatopic control subjects (open circles). Release of both GM-CSF and TNF- α is significantly different between unstimulated and IFN- γ -treated keratinocytes ($p < 0.01$) for both keratinocyte groups. Keratinocytes from patients with AD release, both spontaneously and after incubation with IFN- γ , higher amounts of GM-CSF compared with keratinocytes from control subjects ($p = 0.002$). Basal TNF- α release is enhanced, but not statistically different, in keratinocytes from patients with AD compared with keratinocytes from control subjects. IFN- γ -induced release of TNF- α is much higher in keratinocytes from patients with AD compared with keratinocytes from control subjects ($p = 0.002$). Cytokine quantitation was performed as described in legend to Fig. 4.

keratinocytes from patients with AD were observed to release increased levels of all the cytokines examined in response to different concentrations of IFN- γ (10 to 500 U/ml) and at different time points (24 to 72 hours) (data not shown).

DISCUSSION

In this study keratinocytes cultured from patients with AD and healthy, nonatopic subjects were compared for their sensitivity to some of the proinflammatory effects of IFN- γ . The two groups of keratinocytes did not differ with respect to membrane IFN- γ -receptor expression, and treatment of keratinocytes with IFN- γ determined a dose-dependent induction of HLA-DR and ICAM-1, quantitatively detectable at similar levels in keratinocytes cultured from patients with AD and control subjects. In this regard keratinocytes in AD lesions have been reported to express ICAM-1, especially in the epidermal areas adjacent to more conspicuous dermal T-cell infiltrates but not HLA-DR.^{28, 29} This phenomenon has been explained with the presence of TNF- α , which is sufficient to induce ICAM-1, and the absence of IFN- γ , which is required for the induction of major histocompatibility class II. Our in vitro results confirm that keratinocytes from patients with AD are sensitive to IFN- γ in terms of ICAM-1 and HLA-DR induction and suggest that lack of HLA-DR expression on keratinocytes in some AD lesions can be secondary to a reduced presence of IFN- γ -producing T cells in the skin.

In the following experiments, we examined IFN- γ as a potent inducer of cytokine release by keratinocytes, and we chose to study some of the cytokines known to be involved in the early phases of the epidermal response to inflammatory stimuli (i.e., IL-1 α , IL-1ra, GM-CSF, and TNF- α). IL-1 bioactivity in the skin is finely regulated; keratinocytes can produce IL-1 α , but they cannot efficiently release the active form of IL-1 β .³⁰ Moreover, keratinocytes synthesize, both spontaneously and in response to activation signals, high levels of IL-1ra, which counteracts IL-1 activity by binding competitively to IL-1 receptors without stimulating target cells.^{27, 31} We could measure a 10- to 100-fold excess of IL-1ra versus IL-1 α , either released in the medium or intracellularly, in unstimulated keratinocytes from both patients with AD and control subjects. In these conditions, the intracellular forms of IL-1 α and IL-1ra predominated over the released forms, whereas the intra- and extracellular concentrations were similar after treatment with IFN- γ (data not shown). The ratio between IL-1ra and IL-1 α was always markedly reduced by IFN- γ , although IL-1ra remained in large excess, indicating that IFN- γ tended to favor the predominance of the proinflammatory IL-1 α over its antagonist. In contrast to data previously published,¹⁹ we could follow dependence on IFN- γ dose in the release of all the cytokines tested, including IL-1 α and IL-1ra (data not shown). More important, we could demonstrate that unstimulated keratinocytes from patients with AD tended to release higher levels of IL-1 α , IL-1ra, GM-CSF, and TNF- α compared with keratinocytes from control subjects. However, the differences were statistically significant only for IL-1ra and GM-CSF. After incubation with IFN- γ , the release of all the cytokines investigated was significantly higher for keratinocytes from patients with AD. On the other hand, comparison of IL-1ra/IL-1 α ratio did not show any

significant difference between keratinocytes from patients with AD and control subjects, indicating that an efficient balance is operating on IL-1 bioactivity in the skin of patients with AD. These and previous results on GM-CSF release by keratinocytes from patients with AD and control subjects²³ suggest the existence of an important dysregulation in the synthesis of proinflammatory cytokines by keratinocytes in AD. Interestingly, a recent study on spontaneous cytokine release by epithelial cell cultures obtained from nasal biopsy specimens of different well-characterized types of atopic patients demonstrated that nasal epithelial cells from patients with AD, in the absence of any respiratory involvement, spontaneously released into the culture medium enhanced amounts of IL-8, GM-CSF, and TNF- α when compared with nonatopic control subjects.³² Thus we hypothesize the existence of an intrinsic activation of a broad group of cytokine genes in the epithelial cells of the target organs of atopic disorders, a phenomenon previously attributed only to the effector cells of the immune system.^{23, 32, 33} The reason why IFN- γ stimulation induced exaggerated cytokine synthesis but not increased expression of cell surface molecules in keratinocytes from patients with AD could be related to changes in intracellular signaling or to different gene regulation. IFN- γ signaling pathway involves an early activation of Janus kinases and STAT molecules, which in turn regulate the activity of transcription factors recognizing distinct responsive elements in the promoter region of inflammatory genes.³⁴ It is therefore possible that in keratinocytes from patients with AD, some alteration exists in some step or steps of the late signaling pathway of IFN- γ or, more probably, in the regulatory elements of the cytokine genes, as suggested from previous results²³ and in studies on leukocytes of atopic patients.^{25, 35}

Some reports indicate that IFN- γ could also be very important in the establishment of chronic AD lesions. Indeed, IFN- γ mRNA has been detected in AD lesions and shown to decrease after successful phototherapy,³⁶ and IFN- γ -producing allergen-specific T_{H1} clones have been obtained from chronic skin lesions.^{4, 37} Thus a two-phase model of AD pathogenesis has been proposed, based primarily on the response to epicutaneous application of allergens, in which an initiation phase with a T_{H2}-like inflammatory response and without clinically apparent skin lesions is switched into a second eczematous phase dominated by the presence of IFN- γ .^{38, 39} In this context, IFN- γ can act as a potent stimulus for the synthesis and secretion of proinflammatory cytokines by keratinocytes.

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