Cyclosporin A Rapidly Inhibits Epidermal Cytokine Expression in Psoriasis Lesions, But Not in Cytokine-Stimulated Cultured Keratinocytes

James T. Elder,† Craig Hammerberg,* Kevin D. Cooper,† Takayuki Kojima,* Rajan P. Nair,* Charles N. Ellis,† and John J. Voorhees*

*Department of Dermatology, University of Michigan, Ann Arbor; and †Dermatology Service, Veterans Affairs Medical Center, Ann Arbor, Michigan, U.S.A.

To better understand the cellular target(s) of cyclosporin action in psoriasis, we have studied the effects of systemic short-term (7 d), low-dose (3–7.5 mg/kg) cyclosporin A administration on the expression of the cytokines interleukin (IL)-8 and IL-1β in psoriatic lesions. RNA blot hybridization analysis of pretreatment keratome biopsies revealed that expression of both cytokine mRNAs was highly variable from patient to patient. Significant covariation of both cytokine mRNA levels was noted (r = 0.86, p < 0.0001). However, there was no significant correlation between expression of either cytokine and clinical severity, as measured by the pretreatment Psoriasis Area and Severity Index (PASI). IL-1β protein levels measured by enzyme-linked immunosorbent assay (ELISA) were highly correlated with IL-1β mRNA levels, indicating that the differences in transcript levels accurately reflect differences in epidermal cytokine protein.

The effectiveness of orally administered cyclosporin A (CsA) as an antipsoriatic agent has been well documented [1]. Because CsA appears to be a relatively specific immunosuppressive agent [2], its effectiveness in psoriasis has been cited as evidence for a central role of immune system activation in the pathogenesis of this disease [3,4]. However, at sufficiently high concentrations (2–10 μg/ml), CsA also has significant antiproliferative effects upon cultured human and murine keratinocytes (reviewed in [5,6]), and skin tissue CsA levels approach this range after oral administration of effective antipsoriatic doses [7]. Therefore, in addition to its immunosuppressive actions, CsA might act directly to inhibit the proliferation of keratinocytes.

The psoriatic keratinocyte may also play an important role in the initiation of or maintenance of cutaneous inflammation in psoriasis through the production of chemotactic and proinflammatory cytokines, adhesion molecules, and antigen-presenting molecules such as HLA-DR (reviewed in [6,8]). Because this phenomenon may in turn be elicited by exposure to immune system-derived cytokines, such as interleukin (IL)-1, interferon (IFN)-γ, and tumor necrosis factor (TNF-α), it becomes important to determine whether the effects of CsA on keratinocyte cytokine production in vivo are exerted directly upon the keratinocyte, or instead reflect a blockade of immune system activation.

CsA is an effective antipsoriatic agent at both high [1] and low [7] doses, although the rate of clearing is dose dependent. We have taken advantage of this observation to study the effects of short-term CsA treatment on cytokine gene expression in keratome biopsies of psoriatic skin prior to detectable clinical improvement. This study had two major objectives: i) to determine whether reduced cytokine expression precedes clinical improvement, thus providing some evidence for the participation of epidermal cytokines in psoriasis pathogenesis, and ii) to determine whether the effects of CsA are exerted directly or indirectly on the psoriatic keratinocyte. To accomplish these objectives, we performed Northern blot and enzyme-linked immunosorbent assay (ELISA) analysis of IL-1β and IL-8 expression in keratome biopsies before and after 1 week of low-dose CsA treatment. In addition, we compared the effects of CsA on psoriatic lesions in vitro to its effects on the induction of IL-8 and intercellular adhesion molecule (ICAM)-1 mRNAs by IL-1, IFN-γ, and TNF-α in cultured keratinocytes. The results suggest that amplification of cytokine signaling may occur via a pathway involving keratinocyte-derived IL-8 in psoriatic lesions in vitro. In addition, comparison of in vivo and in vitro results suggests that CsA exerts one or more indirect effect(s) upon the expression of keratinocyte-derived cytokines in psoriasis, presumably by inhibiting the elaboration of activating signal(s) by immune and/or inflammatory cells.

Significant reductions in both cytokine transcripts and in IL-1β immunoreactive protein were noted in the high expression subgroup after 1 week of cyclosporin A therapy, prior to detectable clinical improvement. In contrast to its pronounced effects on epidermal cytokine expression in vivo and the allogeneic mixed lymphocyte reaction in vitro, cyclosporine A did not inhibit the induction of intercellular adhesion molecule (ICAM)-1 or IL-8 mRNAs by cultured keratinocytes in response to IL-1β or the combination of tumor necrosis factor (TNF)-α and interferon (IFN)-γ. These data suggest that epidermal keratinocytes respond to signals produced by activated T cells by coordinate expression of multiple cytokines, and that cyclosporin A acts primarily through blockade of T cells, rather than through keratinocyte activation. Key words: immunology/skin disease/interleukins/ICAM-1/chemokines, J Invest Dermatol 101:761-766, 1993...
Study Design and Tissue Procurement The cohort of 26 patients studied here was a subset of the 85 patients enrolled in a double-blind placebo-controlled trial conducted at the University of Michigan [7]. Patients received oral CsA at doses of 3 mg/kg/d (nine patients), 5 mg/kg/d (12 patients), or 7.5 mg/kg/d (one patient), or placebo consisting of olive oil and polyoxyethylated oleic glycerides (olive oil Labrafail base, four patients). Prior to and after 7 d of treatment, lesional buttocks or thighs' skin was subjected to keratome biopsy at a depth of 0.2–0.4 mm using a Castroviejo keratome. Normal volunteers and psoriatics not treated with CsA were recruited from the Southeast Michigan area and the Department of Dermatology clinic population and biopsied in an identical fashion. All studies involving human subjects were approved by the Institutional Review Board of the University of Michigan, and informed consent was obtained from each patient.

Cell Culture Primary cultures of normal human keratinocytes were prepared as described [9] from keratome biopsies of adult volunteers. Subcultures were expanded in keratinocyte growth medium (KGM; Clonetics, San Diego, CA), and used in the second to fourth passage. Cells were preincubated for 2–16 h with CsA dissolved in dimethylsulfoxide vehicle (0.1% final concentration), with triamcinolone acetonide (TAC) dissolved in 10% ethanol vehicle [10] (0.01% final ethanol concentration) or with vehicle alone. The cell monolayer was then treated with either IL-1 α (5–100 ng/ml; Dainippon, Osaka, Japan) or a combination of TNF-α (20 ng/ml; Amgen, Thousand Oaks, CA) and IFN-γ (100 U/ml; Collaborative Research, Bedford, MA). Cytokine stocks were prepared in sterile phosphate-buffered saline (PBS) and stored in small aliquots at −70°C prior to use. After 6 h of cytokine treatment, cultures were harvested for RNA isolation as described below.

RNA Isolation and Analysis RNA was prepared from snap-frozen keratome biopsies by the guanidinium isothionate-cesium chloride technique as previously described [11], except that cesium trifluoroacetate (Pharmacia, Piscataway, NJ) was used according to the manufacturer's instructions. RNA was isolated from keratinocytes using RNAzol (Tel-Test, Friendswood, TX) as directed by the manufacturer. Forty micrograms total keratocyte RNA, or 20 μg total keratinocyte RNA (determined by optical density at 260 nm) was electrophoretically separated on 1% formamide-agarose gels and transferred to nitrocellulose membrane (Zeta-Tablet; Bio-Rad, Richmond, CA). Blots were hybridized against 32P-labeled probes as described below, and were quantitated using either a laser densitometer [11] or a phosphorimager [12] and normalized to cyclophilin as previously described [11].

Plasmids and Hybridization Protocols Plasmid DNAs were prepared by alkaline lysis and precipitation in polyethylene glycol [13]. cDNA inserts were prepared by digestion with appropriate restriction endonucleases followed by electrophoresis in low–melting-temperature agarose gels. Inserts were 32P-labeled by random priming, and had a specific activity of 1×106 cpm/μg DNA. The IL-1 β [14], lipocortin II [11] and cyclophilin [11] probes used in these experiments have been previously described. The IL-8 probe was a 0.45-kilobase pair (kb) Eco RI insert from the plasmid pPDNCF 2-1 0.5 [15], the ICAM-1 probe was a 1.4-kb XbaI fragment containing the entire ICAM-1 coding sequence derived from the plasmid pGICAM-l [16], and the cellular retinoic acid binding protein (CRABP)-II probe was a 0.9-kb EcoRI fragment derived from a human skin fibroblast library [12]. The 36B4 probe was a 0.7-kb PstI fragment derived from p36B4 [17], which has recently been shown to encode the human acidic ribosomal phosphoprotein PO [18].

Data Analysis All keratocyte biopsy-derived RNA specimens were blotted and sequentially hybridized in parallel, allowing the quantitative data from a total of three blots to be pooled. IL-8, IL-1 β, and CRABP-II densitometry or phosphorimager data were normalized to cyclophilin to control for differences in loading and RNA intactness. Statistical comparisons were made on the normalized data using analysis of variance as previously reported [6].

ELISA Assay of IL-1 α and β In a subset of patients, the week 0 and week 1 keratocyte biopsy specimens were divided, a portion being used to prepare RNA and the remainder to assay IL-1 immunoreactive protein levels. Aqueous extracts of snap-frozen keratomes were prepared using Dulbecco's PBS containing 0.03% polyethylene glycol, and immunoreactive IL-1 β was measured using a commercially available kit (Cistron, Pine Brook NJ) as described [14]. Immunoreactive IL-1 α was quantified using an ELISA composed of monoclonal anti-IL-1 α and polyclonal rabbit anti-IL-1 α (both from Dainippon, Osaka, Japan) as described previously [14].

Immunocytochemistry Portions of the same frozen keratome biopsies used to prepare RNA were mounted in OCT compound (Miles Diagnos-
from pretreatment biopsies of four individuals displaying the highest, and five individuals displaying the lowest, expression of IL-8. There was no detectable difference in the degree of HLe1 staining in the two groups (data not shown). In addition, there was no correlation between the pretreatment expression of these two cytokines and the initial disease severity or eventual clinical outcome, as assessed by the PASI score at weeks 0, 1, 4, and 6 (data not shown).

Coordinate Reduction of Cytokine Expression by CsA In Vivo The Northern blots shown in Fig 3 demonstrate that IL-8 mRNA levels were markedly reduced after 1 week of CsA therapy (Fig 3, second panel from top). IL-1β mRNA levels appeared to be reduced by CsA in a coordinate fashion (Fig 3, third panel from top). Some patients displayed a reduction in the intensity of the cyclophilin control gene after 1 week of CsA therapy. This appeared to be due to reduced RNA intactness, as detected by ethidium bromide staining (Fig 3, top panel). This overall decrease in RNA intactness after CsA was reflected in similar reductions in a separate control gene, lipocortin 2 (Fig 3, bottom panel). These variations were controlled for by normalizing the quantitative data to cyclophilin. Even after normalization, marked and significant reductions in both IL-8 (81.2%, \( p < 0.02 \)) by two-sided paired t test) and IL-1β (85.8%, \( p < 0.015 \)) mRNA levels were observed (Fig 5). In contrast, only a slight decrease in CRABP-II mRNA was observed (8.7%, \( p = 0.05 \)).

Correlation of IL-1β mRNA and Protein Levels To assess the relationship between cytokine mRNA and protein levels, IL-1α and β protein levels were measured by ELISA. IL-1β mRNA and protein levels measured prior to CsA treatment were highly corre-

**Figure 4.** Correlation of IL-1β and IL-8 mRNA levels in pretreatment skin samples. Each point, a different individual, assayed at week 0 of the CsA study. The line indicates the best fit to the data after linear regression analysis \( r = 0.86, p < 0.0001 \).

**Figure 5.** Quantitation of CsA effects on IL-1β, IL-8, and CRABP-II mRNA levels in psoriatic skin. Open bars, week 0; closed bars, week 1. Error bars, SEM (n = 22).
Effects of CsA on IL-1 α and IL-1 β immunoreactive protein (in pg IL-1 per mg keratome extract protein) in psoriatic skin. Week 0 versus week 1 p values: IL-1 β, \( p = 0.004 \), IL-1 α, \( p = 0.77 \). Error bars, SEM.

However, CsA treatment had no effect on IL-1 α protein levels, whereas IL-1 β protein, like IL-1 β mRNA, was significantly reduced after 1 week (\( p < 0.004 \), Fig 6).

**CsA Effects on Cytokine Expression in Cultured Keratinocytes**

Figure 7 shows results representative of 12 experiments in which secondary cultures of normal human keratinocytes were propagated in serum-free KGM, pretreated either with CsA, TAC, or vehicle control, then subjected to treatments with various cytokines. CsA at doses up to 10 \( \mu M \) (12,000 ng/ml) and TAC at doses up to 1 \( \mu M \) (300 ng/ml) were completely ineffective in inhibiting the induction of IL-8 mRNA in response to IL-1 α or IFN-γ plus TNF-α (Fig 7, and additional experiments not shown). Variation of the time of CsA pretreatment from 2–24 h had no effect on the results (data not shown). As previously reported [20–22], ICAM-1 mRNA was induced markedly by IFN-γ plus TNF in keratinocytes grown in KGM. However, pretreatment with either CsA or TAC also had no effect on these responses (Fig 7). IL-1 treatment at 100 U/ml produced only a low and variable ICAM-1 mRNA response, which appeared to be potentiated slightly by CsA (Fig 7 and data not shown).

In additional experiments designed to vary the state of keratinocyte differentiation, cells were cultured in 2 mM CaCl₂ for one passage or allowed to grow to postconfluence prior to CsA pretreatment and cytokine challenge. However, neither of these maneuvers had any effect on the results (data not shown). The lack of effect of TAC was not due to the presence of hydrocortisone in KGM, as its exclusion from the medium for one passage prior to the question of whether the keratinocyte is merely an "innocent

**DISCUSSION**

The strong association of psoriasis with certain HLA types [23], as well as the effectiveness of several antipsoriatic therapies that share immunosuppression as a common feature (e.g., CsA, corticosteroids, methotrexate, UVB, and PUVA [1,24–26]), clearly implicate abnormalities of the immune system in the pathogenesis of psoriasis. CsA and corticosteroids have relatively little effect on keratinocyte proliferation in vitro at clinically relevant doses [5,6,10], suggesting that psoriatic epidermal hyperplasia is not autonomous but rather a response to immune system activation. This concept raises the question of whether the keratinocyte is merely an "innocent
bystander," or in fact plays an active role in the initiation and/or maintenance of the psoriatic lesion.

Keratinocytes may participate in the pathogenesis of psoriasis through the generation of chemotactic signals for immune/inflammatory cells (CD4+ T cells, macrophages, polymorphonuclear leukocytes) [30,32, Fig 2]. IL-8 is a member of the interleukin family of cytokines, which are distinguished by their localization on chromosome 4q21 [33,34]. These cytokines are often considered as secondary response elements of the cytokine cascade, because several of them share the property of inducibility by other (primary) cytokines (namely IL-1 and TNF-α) in a variety of cell types [33,34]. IL-8 also has the properties of a secondary cytokine in cultured keratinocytes, as its expression is induced by IL-1 [21] or by TNF-α acting in concert with IFN-γ [20].

In studying the effect of CSA on epidermal cytokine expression in psoriatic lesions, we analyzed keratocyte biopsies after 1 week of therapy, when no significant improvement in clinical status could be detected. This time interval was selected to avoid the confounding effect of overall reduction in lesion severity on the results. In this study, IL-8 and IL-1 β were variably but coordinately expressed in pretreatment biopsies (Fig 3), resulting in a significant correlation between pretreatment IL-1 β and IL-8 mRNA levels (Fig 4). Curiously, other differences in mRNA ratios did not account for the total number of bone marrow-derived inflammatory cells in the lesions. After 1 week, both cytokine transcripts were markedly reduced (81–85%), whereas CRABP-II transcripts were reduced only slightly (8%) (Fig 5).

Taken together with the high correlation in pretreatment IL-1 β and IL-8 values, their coordinate reductions early in CSA therapy strongly suggest that both cytokine responses are affected by a common proximal stimulus, which is inhibited by CSA.

It is worth noting that all patients, and not just those with high levels of IL-8/IL-1 β expression, rapidly improved with CSA therapy. This suggests either that CSA may inhibit distinct signal transduction pathways in different patients, or that the IL-8/IL-1 β response is variable in space and/or time and is therefore not always detected. We favor the latter explanation, because the differences in pretreatment cytokine mRNA levels between patients could not be accounted for in terms of the total numbers of bone marrow-derived cells in the biopsy specimen. In further support of this concept, one of four placebo-treated patients showed a large increase in IL-8 and IL-1 β mRNAs between weeks 0 and 1, whereas IL-8 mRNA was unchanged in the total number of bone marrow-derived inflammatory cells in the biopsies. This suggests that the keratinocyte is not the direct target of CSA in psoriasis, because the high levels of IL-8 detectable in some lesions indicate that the bulk of the IL-8 expression in psoriatic lesions is occurring in keratinocytes. Although IL-8 can be expressed in a variety of cell types found in the skin, including T cells [35], macrophages [36], and fibroblasts [21], its expression at high levels by keratinocytes in psoriasis has been convincingly demonstrated [32]. In contrast, IL-1 β mRNA levels in psoriatic lesions are near the lower limit of detectability of the Northern blot assay [14], which is approximately 1 copy per cell under the conditions used [37]. Thus, it is unclear whether the IL-1 β mRNA detected by Northern blotting in psoriasis lesions is keratinocyte derived. CSA was unable to inhibit cytokine expression or IL-8 expression in cultured keratinocytes after either CSA or TAC treatment, at concentrations far higher than those required to inhibit the mixed lymphocyte response (Figs 7, 8). This result strongly reinforces the conclusion that the keratinocyte is not the target of CSA action in psoriasis. The inactivity of CSA in this regard was not limited to the IL-8 response, as the ICAM-1 mRNA response to IFN-γ plus TNF-α was unaffected, and the limited ICAM-1 response to IL-1 was slightly potentiated (Fig 7).

These results are consistent with previous studies that show that at concentrations as high as 1–5 μg/ml, CSA does not inhibit, and in fact may slightly potentiate, keratinocyte expression of IL-1, GM-CSF, IF-10, and transforming growth factor (TGF-α, [38,39]).

Regarding the nature of the T-cell-derived signal(s), we speculate that limited expression of primary cytokine(s) either directly by T cells or by infiltrating macrophages and/or dendritic cells under the control of T cells might trigger the expression of IL-8 at much higher levels in nearby keratinocytes. This interpretation would be consistent with the demonstration of clusters of IL-8-expressing keratinocytes directly overlying collections of leucocytes [32]. It is attractive to speculate that one of these primary cytokines might be IL-1 β; however, our previous studies of IL-1 in psoriasis indicated that IL-1 β extracted from psoriatic lesions lacked biologic activity [14]. Among many other possibilities, T-cell–derived IL-8 itself could be one of the signals linking T cells and keratinocytes. Thus, CSA has been shown to inhibit the expression of IL-8 and several related interleukin-α gene family members in T lymphocytes [35] but not in monocytes [35,36]. Alternatively, the relevant signal for the production of IL-8 in keratinocytes could be actual contact with T cells or macrophages themselves [40]. Preliminary reports have suggested that IL-8 can stimulate keratinocyte growth in organotypic culture systems,* and limited effects of IL-8 on keratinocyte proliferation have been observed under serum-free conditions [41]. Whether IL-8 and related cytokines such as gro-α+ directly contribute to psoriatic epidermal hyperplasia in vivo remains to be determined.

The strong positive correlation between IL-1 β mRNA and protein levels before and after CSA therapy indicates that the low level of expression detectable by Northern blotting is reflective of the amount of IL-1 β actually present in psoriatic lesions. However, the biologic significance of these observations remains obscure, and review of the literature reveals many apparent inconsistencies in the levels of cytokine mRNAs, proteins, and biologic activities in psoriatic lesions [14,32,36,42–45]. Potential explanations for these disparate observations include: i) translation of unstable or rare mRNAs into stable proteins; ii) antibody cross-reactions with other keratinocyte proteins; iii) different biologic activities of intra- and extracellular cytokine pools; iv) the presence of specific and non-specific inhibitory proteins; and v) the uptake of cytokines synthesized elsewhere by epidermal keratinocytes. Experimental evidence supports the concept that the skin may serve as a depot for systemic cytokines, including TNF-α [46] and IL-6 [47]. Moreover, proteins extracted from serum albumin (molecular weight 69,000) can enter the epidermal compartment from the circulation [48]. Additional careful investigations will be required to fully elucidate the cellular source(s) of epidermal cytokines in vivo.

Taken together with results demonstrating the coordinate variability in all three forms of MGSA/gro in psoriatic lesions and their reduction after 1 week of CSA treatment, the results presented here indicate that multiple keratinocyte-derived cytokines are under coordinate regulation in psoriasis, perhaps by virtue of a shared signal transduction pathway capable of responding to T-cell–derived signals. The reduction in IL-8/gro chemotactic activity after CSA treatment would be predicted to suppress further influx of T cells and antigen-presenting cells [6,8], contributing to eventual resolution of the lesion. However, substantial clinical resolution required 6–8 weeks of therapy (Fig 1), whereas cytokine expression levels were markedly reduced after only 1 week (Figs 3, 5). It is possible that once initiated, the keratinocyte may be capable of sustaining a hyperproliferative response via CSA-insensitive autocrine mechanisms, such as overexpression of the EGF-like growth factors CSA INHIBITS EPIDERMAL CYTOKINES 765

reduce expression of IL-8 and IL-1β expression by the psoriatic keratinocyte in vivo, and suggest a role for these cytokines in the pathogenesis of psoriasis.

We thank Drs. C. Larsen and K. Matushima for the IL-8 cDNA clone, Dr. Anders Aström for the CRABP-II cDNA probe, Dr. R. Blake Pepinsky for the lipocortin II probe, and Dr. S. W. Caughman for the ICAM-1 cDNA probe. The expert statistical assistance of Ted Hamilton, M.S., and the skilled technical assistance of JFY Chow, Diane Brown, and Qiong Yang are gratefully acknowledged. We are grateful to Dr. Jonathan Barker for many helpful conversations and acknowledge his participation in early experiments on IL-8 expression in psoriatic lesions.

This work was supported in part by the Department of Veterans Affairs (JTE, KDC), USPHS award R29 AR 40016 (JTE), the General Clinical Research Center at the University of Michigan (M01RR0042 from the National Center for Research Resources, National Institutes of Health), and the Babcock Memorial Trust.

REFERENCES