Pimecrolimus Identifies a Common Genomic Anti-inflammatory Profile, is Clinically Highly Effective in Psoriasis and is Well Tolerated

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The ascomycin macrolactam pimecrolimus is a novel inflammatory cytokine release inhibitor that so far has not been administered systemically to humans. In this phase I/II randomized double-blind, placebo-controlled, multiple rising dose proof of concept study psoriasis patients were treated with oral pimecrolimus or placebo. Gene profiling identified a common genomic profile with a downregulation of genes associated with inflammation but no changes in gene expression linked to drug-related side-effects. A steady state of pimecrolimus was reached after 5–10 d, C_{max}, and area under the curve (0–24) was 54.5 ng per ml and 589.9 ng h per ml, respectively, at steady state at the highest dose. There was clear clinical efficacy in patients receiving 20 mg pimecrolimus twice daily and 30 mg twice daily with a reduction of Psoriasis Area and Severity Index by 60% and 75%, respectively. Histopathologically and immunopathologically there was a reversion of the psoriatic phenotype towards normal. There were no notable clinical, laboratory, kidney function, or immunologic side-effects. We conclude that pimecrolimus taken orally is highly effective in a concentration-dependent manner in patients with psoriasis and on a short-term basis it is well tolerated and this is confirmed by its pharmacogenomic profile. The latter also indicates that pimecrolimus should be equally effective in other inflammatory skin diseases. ***Key words: ascomycin/pharmacogenomics/pharmacokinetics/pimecrolimus/psoriasis. J Invest Dermatol 119:876–887, 2002***

Accumulating evidence suggests that psoriasis is an autoreactive, inflammatory disorder based on a chronically ongoing T cell response (Baker and Fry, 1992). This concept is supported by the clinical observation that signs and symptoms of psoriasis can be relieved by systemic immunosuppressive therapy employing cyclosporine A (Ellis *et al*, 1986; Mihatsch and Wolff, 1992), tacrolimus (Jegasothy *et al*, 1992), systemic monoclonal antibodies, or fusion proteins directed to CD3 (Weinshenker *et al*, 1989) and CD4 (Nicolas *et al*, 1991; Prinz *et al*, 1991; Morel *et al*, 1992; Bachelez *et al*, 1998; Gottlieb *et al*, 2000b), CD_{11a} (Gottlieb *et al*, 2000a), the high-affinity interleukin (IL)-2 receptor, CD2, and tumor necrosis factor (TNF)-α (Gottlieb *et al*, 1995a; Krueger *et al*, 2000; Mrowietz *et al*, 2000; Oh *et al*, 2000; Owen and Harrison, 2000; Chaudhari *et al*, 2001; Ellis and Krueger, 2001). The ascomycin macrolactam derivative pimecrolimus is a novel selective inflammatory cytokine release inhibitor (Grassberger *et al*, 1999; Meingassner *et al*, 1997; Neckermann *et al*, 2000; Paul *et al*, 2000; Wellington and Spencer, 2000), which has been shown to suppress disease activity in atopic dermatitis (Van Leent *et al*, 1998; Harper *et al*, 2001), contact dermatitis (Queille-Roussel *et al*, 2000), and psoriasis (Mrowietz *et al*, 1998) when applied topically. Pimecrolimus has never been administered systemically to humans and as, in animal models, it exhibits high anti-inflammatory activity after systemic application (Meingassner *et al*, 1997; Stuetz *et al*, 2001)[1] and combines this with a low potential for systemic immunosuppression (Stuetz *et al*, 2001) we addressed the question whether pimecrolimus could be considered a drug for the systemic treatment of patients with psoriasis and possibly other inflammatory skin diseases. Here we report on a randomized, double-blind, placebo-controlled proof of concept phase I/II study that evaluates the pharmacogenomic and pharmacokinetic profile and the efficacy, safety, and tolerability of this drug as an oral treatment in psoriasis. To the best of our knowledge, this is the first study that includes pharmacogenomic profiling of blood cells during therapy.

MATERIALS AND METHODS

Study design In this randomized, double-blind, placebo-controlled, multiple rising dose study patients were screened between days –21 and

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[1] Meingassner JG, Fahrngruber H, Bavandi A: SDZ ASM 981, in contrast to CyA and FK 506, does not suppress the primary immune response in murine allergic contact dermatitis. J Invest Dermatol 114: 832, 2000 (Abstr.)

–2, baseline examinations were performed on days –2 and –1, and treatment was given from days 1 to 28. Patients were hospitalized for study weeks 1 and 2 and were further closely monitored as outpatients on a daily basis during study weeks 3 and 4. From days 27 to 30, patients were hospitalized again for pharmacokinetic profiling. Outpatient follow-up was done on day 34 and at completion of study between days 40 and 42.

Five consecutive cohorts of 10 patients each were treated with rising doses of oral pimecrolimus (SDZ ASM 981, Elidel®, provided by Novartis Pharma AG, Basel, Switzerland) for 4 wk (eight patients in each cohort) or with placebo (two patients in each cohort). Escalating doses were 5 mg once daily, 10 mg once daily, 20 mg once daily, 20 mg twice daily, and 30 mg twice daily. Start of the next higher dose level was permitted only after demonstration of tolerability and safety at the preceding lower dose. After discontinuation of treatment, patients were followed-up for 2 wk with further follow-up at varying intervals up to 12 mo. All patients gave written informed consent and the protocol was approved by the institutional review board. The study was conducted according to the Declaration of Helsinki principles.

Table I. Demographics

	Pimecrolimus	Placebo
Subjects entered	40	10
Completed	37[a]	10
Male/female	40/0	9/1
Mean age (y) [range]	39.5 [22–61]	42.2 [19–60]
Body weight (kg) [range]	82.9 [55–108]	81.2 [60–99]
PASI at baseline [range]	21.2 [10.4–44.4]	22.2 [10–44.4]

[a]One patient treated with 10 mg once daily pimecrolimus withdrew his consent after 21 d of treatment. One patient on 30 mg twice daily did not complete the full PK profile on the 28th day and had to be excluded; one patient on 30 mg twice daily withdrew on day 24 for external reasons.

Patients We studied 50 patients older than 18 y of age with moderate-to-severe chronic stable plaque type psoriasis for at least 3 y. The severity of the psoriasis was assessed by using the Psoriasis Area and Severity Index (PASI). A minimum PASI of 10 was required for inclusion. Psoriasis should have been stable during the 2 wk prior to entry into the screening phase and should require a systemic treatment according to the investigator's judgement. Erythrodermic or pustular psoriasis were excluded. All patients had no other important concurrent diseases nor a history thereof and had not been treated with immunosuppressive agents, cytostatics, systemic corticosteroids, or radiation therapy within 6 mo; psoralen + ultraviolet A photochemotherapy, ultraviolet phototherapy, or other disease modifying drugs for 1 mo; and topical therapy for 1 wk. Other exclusion criteria were of cytochrome-inducing or inhibiting and nephrotoxic drugs prior to study; positive serology for hepatitis B, C, and HIV serology as well as a history of alcohol or drug abuse. The baseline characteristics and measures of disease activity were similar in the treatment and placebo groups (**Table I**). Forty-seven of 50 patients completed the study; three patients withdrew or had to be excluded after 21, 24, and 28 d, respectively, of treatment for reasons that were not related to therapy or adverse effects.

Gene profiling Blood samples were collected from patients in the cohort receiving 30 mg pimecrolimus twice daily before and after 13 or 14 d of treatment and submitted to gene expression analysis. Extraction of RNA from whole blood samples, the preparation of the target, hybridization, and analysis of the arrays were performed as recommended by the Expression Analysis Technical Manual (Affymetrix Inc., Santa Clara, CA). Good quality total RNA was used to synthesize double-

Table II. Genes consistently modulated in pimecrolimus treated patients[a]

Pathway/functions	Gene	Variation	Description
Ascomycin target pathway	Macrophilin-12	↓↓	Cytosolic drug receptor
	Calmodulin	↓↓	Regulation of intracellular calcium signaling T cell activation pathway
	Calcium modulating cyclophilin ligand CAMLG	↓	T cell activation pathway downstream of the TCR and upstream of calcineurin
	NRGN Neuragranin	↓	Storage and release of calmodulin and calcium
Antigen presentation	Ii Invariant chain, CD74	↓↓	Associated with HLA class II molecule
	HLA-E	↓↓	Class I like molecule recognized by natural killer lymphocytes
	HLA-F	↓↓	Class I like molecule: Induced by IFN-γ
	4-1BB ligand	↑	Stimulates $CD8^+$ T cells
Cellular metabolism	Ribosomal proteins (> 50)	↓ to ↓↓↓	
	Nascent polypeptide associated complex	↓	Ribosomes binding to the reticulum membrane
	Histones H3.3 and H2	↓/↓↓	Chromatin structure
	Cyclin D2 and D3	↓↓↓/↓↓	Progression in G_1 phase
	Zinc finger protein ZNF141	↑	Anti-proliferative properties
Transcription factor, signaling	EF-1	↓↓↓	Elongation factor 1 δ
	HMG-17	↓↓↓	DNA binding protein nonhistone
	TF Iia	↓↓	Transcription factor
	Tyrosine phosphatase	↓	Signal transduction
	G gamma 11	↓	Protein G signaling pathway
Chemotaxis and leukocyte migration	L-selectin	↓↓	Leukocyte rolling. Migration
	LFA1a/LFA1b	↓↓/↓↓	Migration at the inflammation site.
	MIC-2 CD99	↓↓↓	Regulates LFA-1/CAM-1 adhesion of lymphocytes
	RANTES	↓↓↓	Chemoattractant for lymphocytes, monocytes, eosinophils, basophils. T cell proliferation activation, T helper 1 differentiation. Release of inflammatory mediators
	Platelet factor activation 4	↓	Leukocyte recruitment, histamine release
	Selectin P ligand SELPLG	↓↓	Interacts with P and L-selectins. CLA
Inflammation	Leukotriene A_4 hydrolase	↓	Responsible for the synthesis of leukotriene B
	FC-epsilon binding protein	↓↓↓	Galectin 3 release of inflammatory mediators
	Thromboxane A_2 receptor	↓↓	Smooth muscle contraction
	Prostaglandin endoperoxide synthase 1	↓↓↓	First step of leukotriene and thromboxane synthesis
	Kallistatin	↑	Inhibits kallikrein kininogenase

[a]Median fold change for the seven treated patients: from –2 fold to –5 fold: ↓, from –5.1 to –10 fold: ↓↓, from –10 to –30: ↓↓↓, from –30 to –100: ↓↓↓↓, from +2 to +5: ↑

Table III. Selected genes not modulated in pimecrolimus-treated patients

Pathway/function	Gene
Lymphocyte markers[a]	CD3 δ, CD3 epsilon, CD8, granzyme B, granzyme A
Cytokines[b]	IL-1, IL-2Rγ, IL-3R, IL-4R, IL-7, IL-8, IL-8R, IL-10, transforming growth factor β1–3
Apoptosis pathway and stress response	CASP, FADB-like apoptosis regulator, caspase 2, 3, 4, 6, 7, 8, 10, Requiem, BCL-2 interacting killer, ubiquitin-like domain, member 1, stress-induced phosphoprotein 1, microsomal glutathione S-transferase 2, esterase D, glutathione S-transferase π, glutathione S-transferase M4, glutathione peroxidase 4

stranded cDNA using the Superscript Choice System (Life Technologies, Grand Island, NY), the cDNA was then *in vitro* transcribed (MEGA Script™ T7 kit, Ambion, Huntingdon, UK) to form biotin-labeled cRNA. Labeled cRNA was hybridized to probe arrays for 16 h at 45°, washed according to the EukGE-WSZ protocol (Affymetrix Inc.) and stained with streptavidin–phycoerythrin conjugate. Arrays were scanned twice with the Gene Array® scanner (Affymetrix Inc.). The data were analyzed using the NPGN (Novartis PharmacoGenomic Network, Novartis Pharma AG, Basel, Switzerland) data base and GeneSpring® (Silicon Genetics, Redwood City, CA). The genes consistently modulated in pimecrolimus-treated patients were sorted to build a profile.

Pharmacokinetics Pimecrolimus blood concentrations were determined by radioimmunoassay (Queille-Roussel *et al*, 2000) in all cohorts immediately before the morning dose on days 4, 6, 8, 10, and 21, during the washout period on days 30 and 34, and at study completion (between days 40 and 42). Full profiles (times 0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h postdose) were performed on days 1, 13, and 28.

Efficacy assessments The efficacy of SDZ ASM 981 was assessed by means of the PASI score and by a global evaluation of the status of the disease performed by the investigator.

The PASI was calculated by using the following procedures. The head, trunk, upper limbs, and lower limbs were assessed separately for erythema, infiltration, and scaling. The average degree of severity of each symptom in each of the four body parts was assigned a score of 0–4. The area covered by lesions on each body part was estimated as a percentage of the total area of that particular body part and a score from 0 to 6 was then assigned. The total score for each body part was obtained by multiplying the sum of the severity scores of the three basic lesions by the area score, then multiplying the result by the constant weighted value assigned to that body part. The sum of the scores of the individual body parts gives the PASI, which, theoretically, can range from 0 to 72.The area evaluation for calculating PASI took into account the true surface affected by psoriasis. Whenever possible, the PASI was assessed by the same investigator for each subject.A global evaluation of the status of the disease was rated by the investigator using the following qualitative scale: totally clear (normal skin), almost clear, mild, mild–moderate, moderate, moderate–severe, and severe. For documentation all patients were photographed on days 0, 7, 14, 28 (or 29), and at completion of the study. Whole body photographs and photographs of three selected (indicator) lesions were taken on each occasion.

The PASI was determined at screening, at baseline, on days 7, 14, 28 (or 29), and at completion of study (days 40–42). A global dermatologic evaluation was performed at baseline, on day 28 (or 29) at completion of study, and at follow-up visits.

The referenced value for the PASI and the global evaluation of the status of the disease was the value assessed at baseline.

Safety and tolerability assessments Physical examination, blood pressure, heart rate, and electrocardiogram were performed and body weight was determined at screening, baseline, on days 2, 4, 8, 14, 21, 28, and at study completion. Laboratory safety tests were done at screening, at baseline, on days 2, 4, 8, 14, 21, and 28 and at study completion. They included full blood biochemistry, hematology, and urine analysis. Glomerular filtration rate (inulin clearance) and renal plasma flow (paraamino hippuric acid [PAH] clearance) were performed at baseline, on days 13 and 28, and at completion of the study; and glucose tolerance tests at baseline and on day 28 for the 20 mg twice daily and 30 mg twice daily cohorts.

Immunologic investigations Skin tests (Immignost®) for delayed type hypersensitivity to a battery of recall antigens (tetanus toxoid, diphtheria toxoid, streptococcal antigen, tuberculin, *Candida albicans* antigen, *Trichophyton mentagrophytes*, and *Proteus mirabilis* antigens) were performed in the 30 mg twice daily cohort at screening or within the first week of treatment and within 2 mo after completion of the study. Tests were performed on the volar aspect of the lower arm and repeat tests were done on the other arm. Tests were read at 48 h and 72 h and redness plus palpable induration were considered a positive reaction. For

Table IV. Expression of lymphocyte genes in pimecrolimus treated patients

	Fold changes				
Patient no.	Granzyme B	Granzyme A	CD8 β	CD3 ε	CD3 δ
45	+3.4	+3.1	+3.4	+1.9	=
49	=	=	–1.7	=	=
41	+4.1	+2.6	–1.2	+1.9	–7
48	–2.4	–1.7	–3.4	+1.7	–1.9
46	+4.2	+2.3	+2.4	+2.3	+1.5
50	+4.5	+1.6	+1.3	+1.9	+1.1
42	=	+1.6	=	–1.1	–3.5
44	+10.8	ND	ND	ND	ND
Median	+4.9	+1.6	+2.4		
Proportion of responders	5/8	5/8	3/8		

ND, nondetermined (data reported as non detected in the array data).

Table V. Other, selected early cytokine, and cytokine receptor genes not consistently modulated in pimecrolimus-treated patients

	No. of patients			
	Up-regulated	Down-regulated	Unchanged (fold change < 2)	Placebo Patient no.
IL-1 α	3	3	1	Unchanged
IL-1 receptor antagonist	1	3	3	1 downregulated, 1 unchanged
IL-2 receptor γ	1	0	6	Unchanged
IL-3 receptor α (low affinity)	3	1	3	1 upregulated, 1 unchanged
IL-4 receptor	2	1	4	Unchanged
IL-7 receptor	3	2	2	Unchanged
IL-8	3	2	2	Unchanged
IL-8 receptor α	2	4	1	1 downregulated, 1 unchanged
IL-8 receptor β	3	1	3	Unchanged
IL-9 receptor	3	1	3	Unchanged
IL-10	4	0	3	Unchanged
IL-10 receptor α	0	1	6	1 downregulated, 1 unchanged
IL-10 receptor β	0	4	3	1 downregulated, 1 unchanged
IL-14	1	4	2	2 downregulated
IL-17	1	4	2	Unchanged

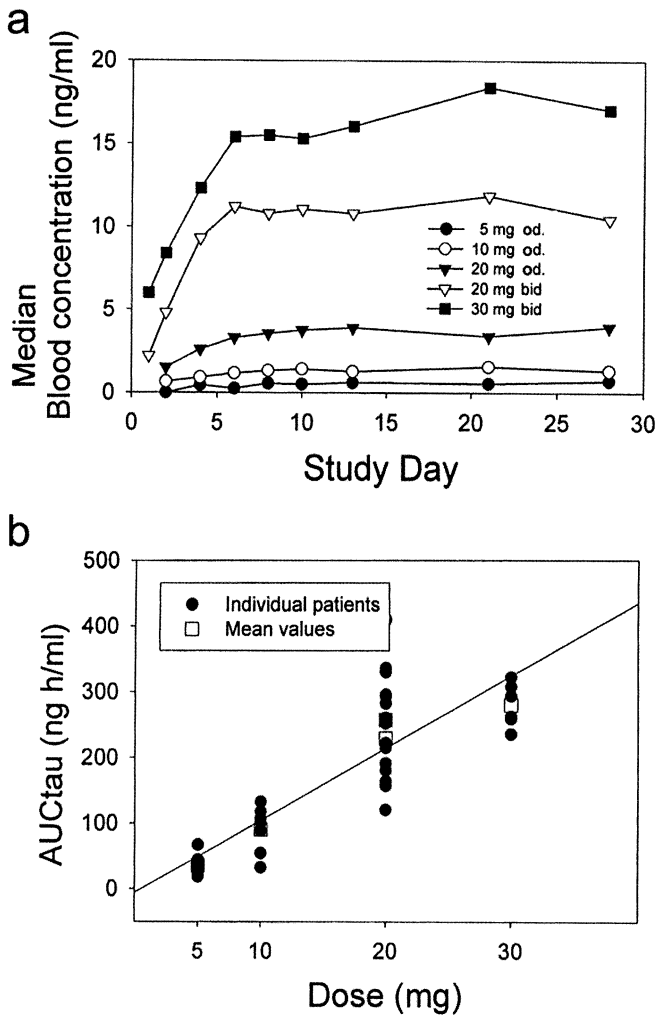


Figure 1. (*a*) Median blood trough concentrations of pimecrolimus throughout the study. Blood concentrations of pimecrolimus increased in proportion to the dose and steady state as reached after days 5–10. (*b*) AUC_τ *vs* dose on day 28. AUC_τ increased in proportion to the dose.

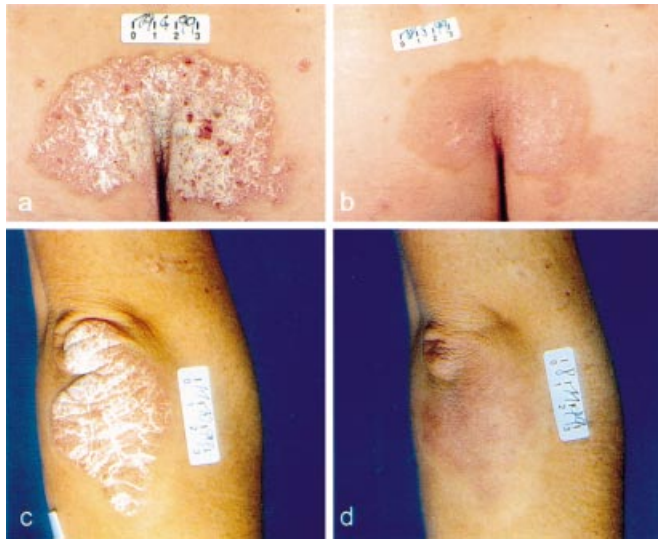


Figure 2. Clinical efficacy of pimecrolimus is shown for a patient receiving 20 mg twice daily photographed on day 0 (*a*) and day 28 (*b*) and for a patient receiving 30 mg twice daily on day 0 (*c*) and day 28 (*d*).

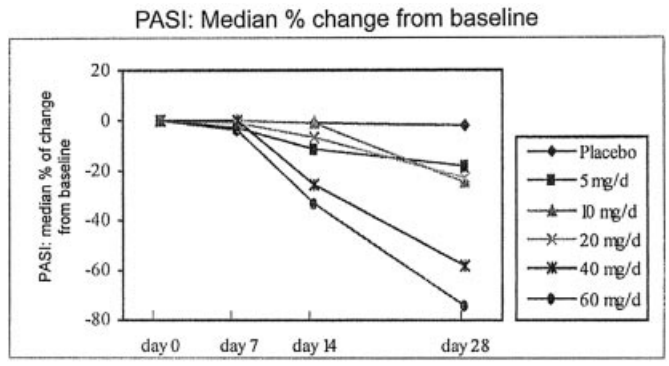


Figure 3. Clinical efficacy of pimecrolimus as assessed by percent PASI change from baseline for the different dose levels. Efficacy is clearly dose-related and reflects as PASI reduction of 60% and 75%, respectively, on day 28 for doses of 20 mg twice daily (40 mg per day) and 30 mg twice daily (60 mg per day).

each patient and at each reading the number of positive reactions was recorded and the diameters of test sites were measured and recorded in millimeters.

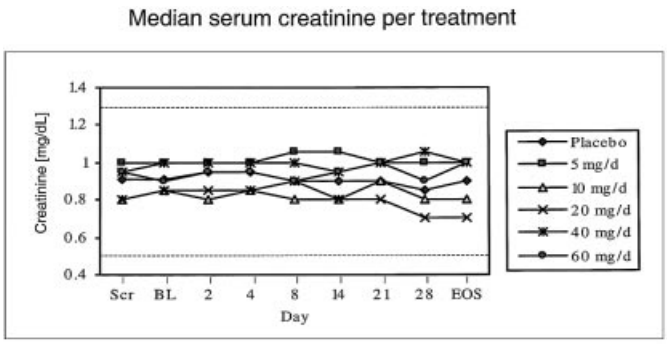
Quantitative determination of lymphocyte subpopulations in the peripheral blood were done by flow cytometry and T lymphocyte proliferation in response to *ex-vivo* stimulation with superantigens and mitogen and cytokine production after *ex-vivo* stimulation were also assessed. The blood samples were taken before treatment and after 4 wk of treatment (day 29) with 20 mg once daily, 20 mg twice daily, or 30 mg twice daily pimecrolimus or placebo. For immunofluorescence analysis a whole blood lysis method (Becton Dickinson, Erembodegem, Belgium) was performed using directly labeled monoclonal antibodies to CD3 (Becton Dickinson), CD4 (Dako AS, Glostrup, Denmark), CD8 (Dako), CD25 (Dako), CD56 (Becton Dickinson), CD19 (Dako), T cell receptor (TCR) αβ (Immunotech, Marseille, France), TCR-γδ (Immunotech), pan major histocompatibility complex class II (Dako), and CD45 (Dako). The samples were analyzed on a FACSCalibur instrument using the Paint A software (Becton Dickinson). Positive lymphocyte gating strategy was based on scatter analysis and CD45 positivity, and calculated also in absolute numbers based on a blood differential count obtained from the same blood sample. CD4, CD8, and expression of the activation markers CD25 and major histocompatibility complex class II were measured on CD3-positive lymphocytes.

For cell proliferation studies blood was drawn into a syringe prefilled with stabilizator free heparin (final concentration 50 U per ml) and diluted 1 : 10 with X-VIVO 15 medium (BioWhittaker, Walkersville, MD) supplemented with L-glutamine (Life Technologies). Two hundred microliter aliquots of diluted whole blood were stimulated in U-bottomed microtiter plates (Nunc, Roskilde, Denmark) in the presence of a mixture of the superantigens SEA plus SEB (Sigma, St Louis, MO; each at final concentrations of 250 ng per ml and 62.5 ng per ml), phytohemagglutinin (PHA) (Sigma; 4 μg per ml) or medium for 5 d. During the final 16 h of incubation the cultures were labeled with 1 μCi

Table VI. Summary of adverse events[a]

	0 mg	5 mg	10 mg	20 mg	40 mg	60 mg
Feeling of warmth		1		1	3	7
Viral infection		1			1	1
Local infection				1		
Pruritus	1		1			
Headache	1	1				1
Vertigo/malaise	1			1		1
Paraesthesia					1	
Light sensitivity					1	

[a]The only consistent adverse effect was a feeling of warmth. The viral infections were episodes of common cold.



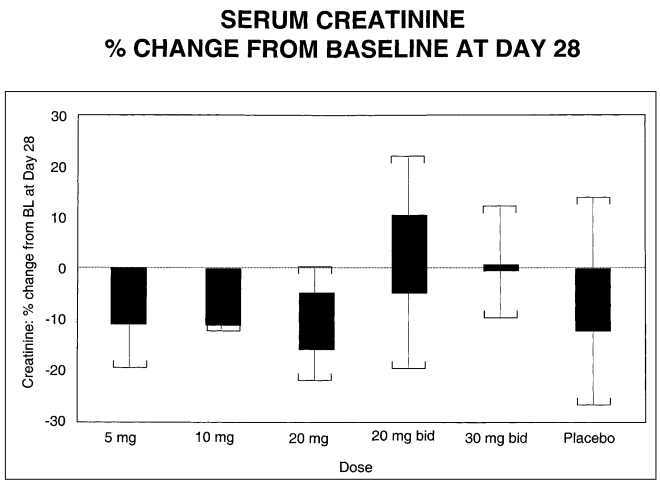


Figure 4. (*a*) Median serum creatinine levels in the placebo and various pimecrolimus [5 mg, 10 mg, 20 mg, 20 mg twice daily (= 40 mg per day) and 30 mg twice daily (= 60 mg per day)] dose groups. Serum creatinine never increased more than 30% compared with baseline. There are no notable changes in the creatinine levels from screening to end of study (EOS) scr: screening visit, BL: baseline. (*b*) Percent change of serum creatinine from baseline at day 28 in the different dose groups and placebo. The highest percent change from baseline was seen in the 20 mg twice daily dose group, which exceeded that of the 30 mg twice daily dose group that showed fewer changes than the placebo group.

^{3}H-thymidine (NEN, Boston, MA) and the incorporated radioactivity was measured using the Wallac Microbeta MeltiLex scintillation system.

For cytokine production a 1 ml aliquot of 1 : 10 diluted (X-VIVO 15 medium) whole blood was stimulated for 48 h with phytohemagglutinin (4 μg per ml) plus phorbol myristate acetate (PMA; 10^{-7} M; Sigma) in round-bottomed 15 ml tubes (Becton Dickinson), thereafter the supernatant was analyzed for the presence of IL-2, IL4, IL-10, and interferon (IFN)-γ, and -α using standard sandwich enzyme-linked immunosorbent assay methodology using commercially available monoclonal antibody pairs (Pharmingen, San Diego, CA).

Biopsies Punch biopsies of lesional skin at baseline and on day 28 in patients of the 20 mg once daily, 20 mg twice daily, and 30 mg twice daily cohorts with pimecrolimus or placebo were evaluated for the histomorphologic and immunohistochemical phenotype of psoriasis, epidermal thickness, the state of differentiation, activation and proliferation of keratinocytes, and the inflammatory infiltrates employing monoclonal antibodies to CD1a, CD3, CD4 (Dako AS, Glostrup, Denmark), CD8, keratin-16, Ki-67, HLA-DR (Neomakers Inc., Union City, CA) filaggrin, and involucrin (Biomedical Technologies Inc., Stoughton, MA). For staining a tech made horizon™ (Dako) and for evaluation a Zeiss Axioscope microscope using a color 3 chip camera and a KS 400 3.0 image analysis system (Zeiss Viscon, Oberkochen, Germany) were employed.

Statistical analyses Demographic variables were analyzed by descriptive statistics and the numerical data of the safety assessments at

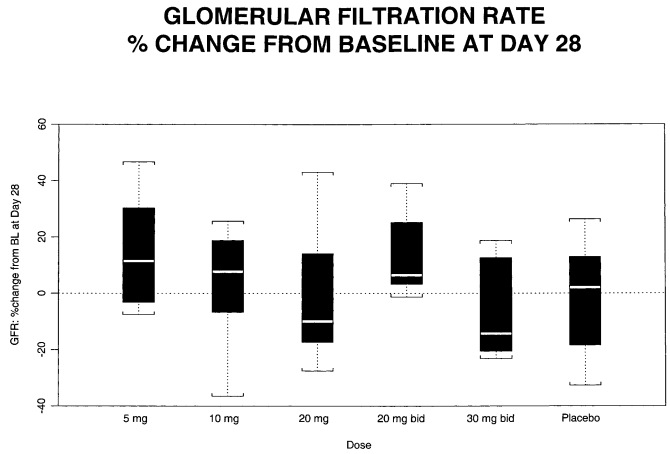


Figure 5. Glomerular filtration rates. Percent changes from baseline BL at day 28 for the different dose groups and placebo. There are no differences between pimecrolimus and placebo-treated patients. bid: twice daily.

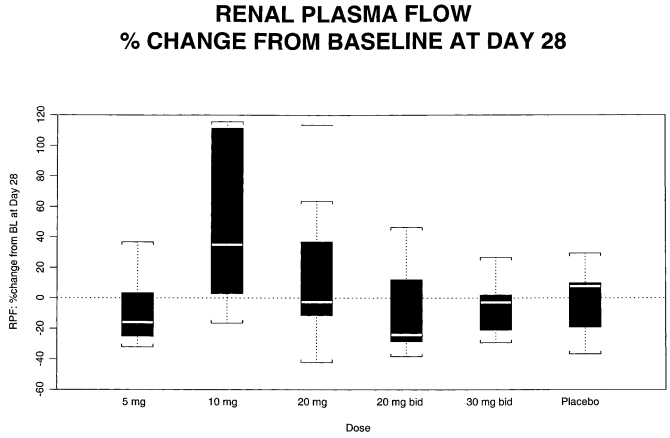


Figure 6. Renal plasma flow. Percent changes from baseline (BL) at day 28 for the different dose groups and placebo. There are no significant differences between the 5 mg, 20 mg twice daily, and 30 mg twice daily pimecrolimus group and placebo-treated patients. The increase of renal plasma flow in the 10 mg pimecrolimus group is unexplained. bid: twice daily.

each evaluation time were calculated over all subjects. Pharmacokinetic parameters [C_{max}, t_{max}, area under the curve $(AUC)_{0-24\ h}$, trough concentration] were derived by standard noncompartmental analysis methods from the concentration–time profiles measured on days 1, 13, and 28. Attainment of steady state was evaluated by a linear regression of log-transformed trough concentration *vs* day; drug accumulation by a paired t test of log-transformed trough concentration and AUC_τ (area under the concentration–time curve during a dosing interval [τ] at steady state); and dose-proportionality by an ANOVA on log-transformed dose-normalized C_{max} and AUC_τ.

Data collected on the PASI score were tabulated by dose and appropriate summary statistics were performed on percentage of change from baseline. For statistical evaluation of the inflammatory infiltrate in skin sections we used the SPSS 8.0 software for Windows. Data collected for each cell population were analyzed using the paired Student's t test.

For the immunologic data in the peripheral blood, an ANOVA was performed on the log-transformed ratio of data before and after treatment. In addition, the change in the parameters before and after treatment with pimecrolimus was compared with the relative change in the placebo group. The software package SAS (Release 6.12 for Windows-NT, SAS Institute Inc.) was used for data analysis.

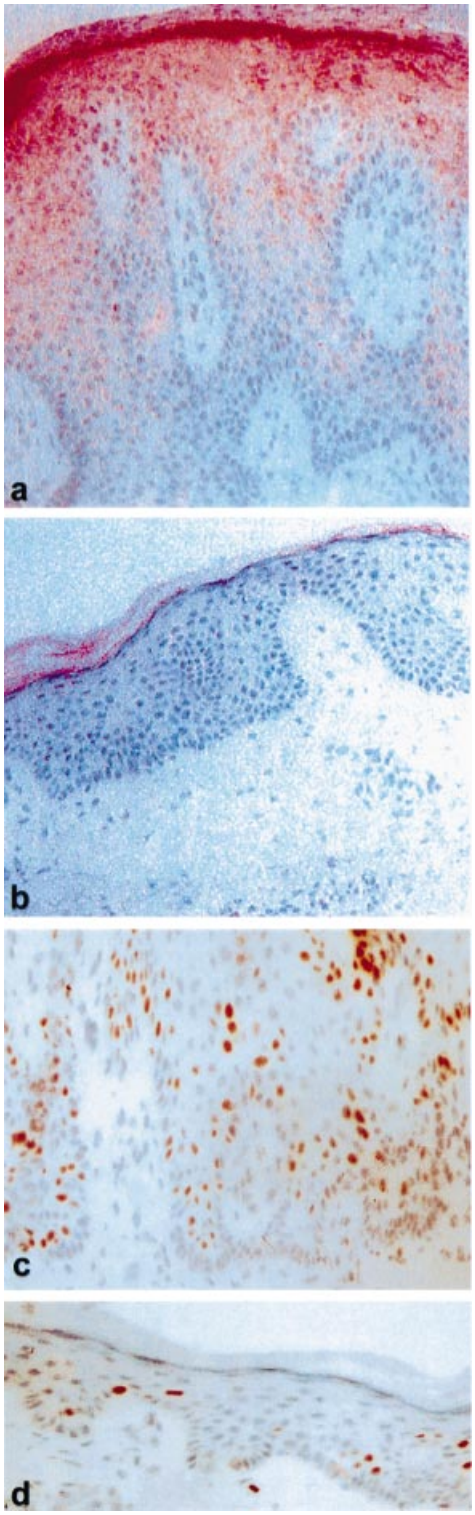


Figure 7. Skin biopsy of a patient receiving 30 mg pimecrolimus twice daily at baseline (*a*) and day 28 (*b*). Section stained for the epidermal proliferation-associated keratin-16 (red). Note significant reduction of epidermal thickness, inflammatory infiltrate, parakeratosis, and loss of keratin 16 staining at the end of the treatment period. This is a reversal of the psoriatic phenotype to almost normal. (*c*) Shows a section stained for proliferating Ki-67$^+$ keratinocytes. Note significant reduction of Ki-67$^+$ nuclei at end of treatment (*d*) that accompanies the reduction of acanthosis to that of a normal looking epidermis.

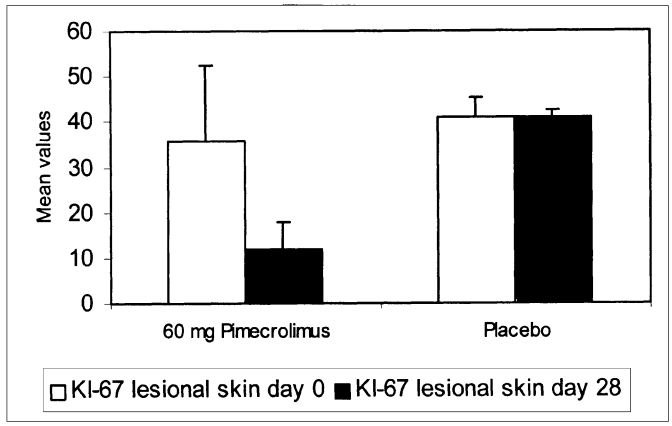
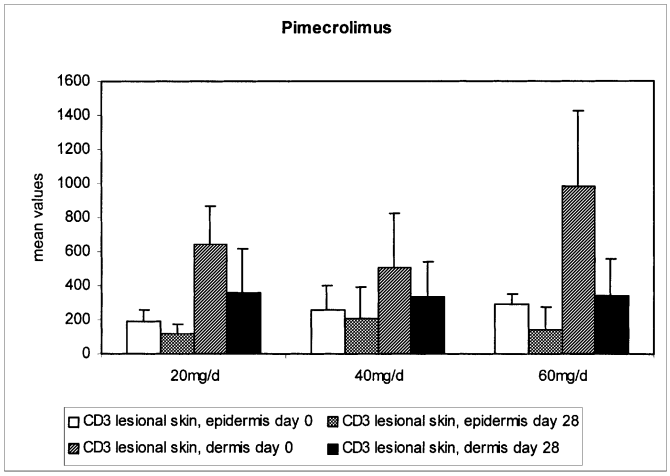


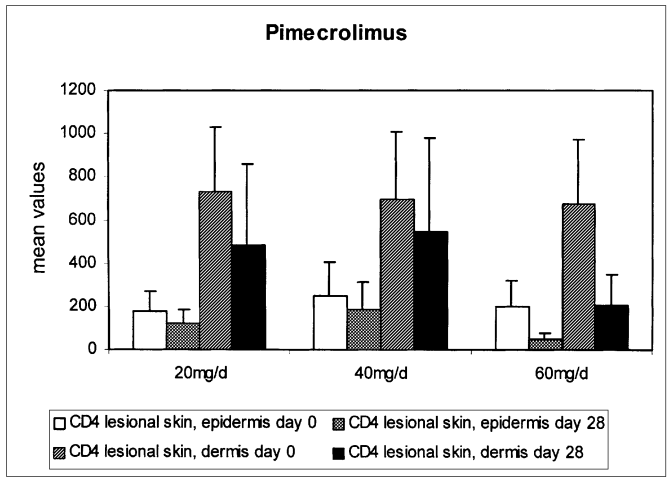
Figure 8. Ki-67 proliferating keratinocytes at baseline (day 0) and day 28 in a 30 mg twice daily (60 mg) pimecrolimus and placebo-treated patient. Note the significant reduction of Ki-67 positive cells ($p = 0.02$) under pimecrolimus as compared with placebo.

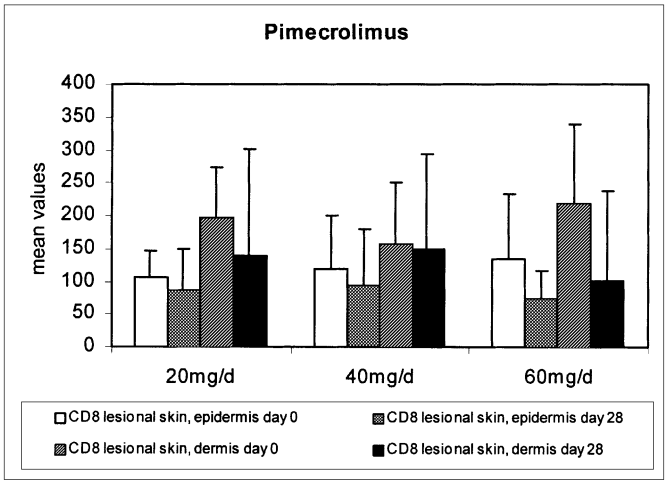
RESULTS

Gene expression analysis identifies a common genomic profile Blood samples subjected to gene expression analysis using gene chips that allow the survey of 7129 genes permitted the identification of a common genomic profile of pimecrolimus consisting of about 150 genes. A large majority of those genes was downregulated and sorted into functional categories (**Table II**). Pimecrolimus strongly downregulated the expression of genes associated with the macrolactam target pathway (e.g., macrophilin-12, calmodulin), cellular activation and proliferation (histone 2, histone 3.3, cyclin D2), antigen presentation (Ii invariant chain, CD74), to cellular metabolism, signal transduction, transcription factors, and inflammatory mediators, such as leukotriene A_4 hydrolase, prostaglandin endoperoxide synthase, and Galectin 3 (**Table II**). Kallistatin, an inflammation regulating protein, was upregulated (**Table II**). Genes related to lymphocyte recruitment, chemotaxis, and cellular migration (LFA-1, CLA, and L-selectin) were also downregulated. No significant changes in T lymphocyte markers (CD3, CD8, and granzymes) could be detected at the RNA expression level suggesting an absence of a systemic T cell suppressive effect of the drug (**Tables III and IV**). In addition, no change of expression was seen for transforming growth factor β1–3 (**Table III**) and for IL-1, IL-2, IL-8, and IL-10 (**Tables III** and **V**). None of the genes that changed expression were clearly related to side-effects or toxicity, and genes associated with apoptosis, stress, and enzymatic induction were not changing expression (**Table III**). Of course, data presented here pertain to blood only as internal organs were not biopsied. For reasons of space the details of these investigations will be published separately in detail.

Pharmacokinetics of pimecrolimus reveal rapid absorption, attainment of steady state after 5–10 d, and linear dose dependency at steady state Pimecrolimus was rapidly absorbed; median t_{max} was between 0.8 and 2.0 h across all the dose groups and collection days. Both C_{max} (once daily dose groups) and $AUC_{(0–24)}$ (both once daily and twice daily groups) increased broadly in proportion to the dose (**Fig 1**). Some statistically significant deviations from ideal dose proportionality were observed, but these were of a minor nature and may have resulted from the high variability observed in the small number of patients per group. Steady state was reached after days 5–10 and at daily administration of 30 mg twice daily, C_{max} and $AUC_{0–24}$ reached 54.5 ng per ml and 589.9 ng h per ml at steady state, respectively (**Fig 1**). In the 30 mg twice daily cohort, median trough concentrations increased from 6 ng per ml on day 1 to about 15 ng per ml at steady state (**Fig 1**).







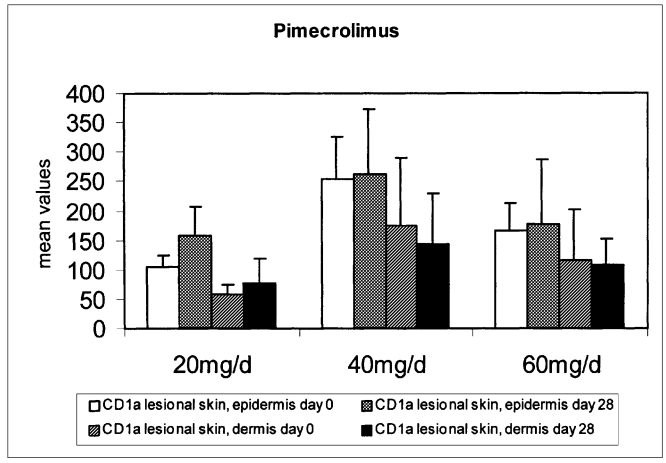


Figure 9. Reduction of lymphocytic infiltrate and unchanged numbers of CD1a$^+$ cells (Langerhans cells) during pimecrolimus treatment. (*a*) CD3$^+$ cells in psoriatic epidermis and dermis at days 0 and 28 at dose levels of 20 mg, 40 mg (= 20 mg twice daily), and 60 mg (= 30 mg twice daily). The reduction of CD3$^+$ cells at dose levels of 20 mg in the epidermis ($p = 0.009$) and dermis ($p = 0.04$) and of 60 mg (= 30 mg twice daily) in the epidermis ($p = 0.05$) and dermis ($p = 0.006$) are significant. (*b*) CD4$^+$ cells are significantly reduced at day 28 both in the epidermis ($p = 0.02$) and dermis ($p = 0.01$) at the 60 mg (30 mg twice daily) dose level of pimecrolimus. (*c*) Pimecrolimus reduces CD8 cells in epidermis and dermis, at all dose levels. The reduction of CD8$^+$ cells in the dermis at 60 mg (30 mg twice daily) is significant ($p = 0.077$). (*d*) Pimecrolimus does not significantly affect CD1a$^+$ cells in the epidermis and dermis at any of the dose levels of pimecrolimus (e.g., 60 mg per day epidermis $p = 0.958$; dermis $p = 0.887$).

Pimecrolimus is highly effective in suppressing psoriasis Whereas there was no change in disease activity and PASI scores in the patients on placebo and the cohorts receiving once daily pimecrolimus at 5 mg, 10 mg, and 20 mg, there was clear efficacy in the cohorts receiving 20 mg pimecrolimus twice daily and 30 mg twice daily. This involved a reduction of scaling, erythema, and infiltration of lesions and led to a reduction of PASI score and even to an almost complete or complete clearing of lesions on day 28 (one patient in the 20 mg twice daily and two patients in the 30 mg twice daily cohorts) (**Fig 2**). This response was clearly concentration related as shown in **Fig 3**. The mean reduction of PASI (change from baseline at day 28) was 60% for the 20 mg twice daily and 75% for the 30 mg twice daily cohorts (**Fig 3**).

Pimecrolimus is well tolerated and lacks notable clinical and laboratory side-effects There were no clinically notable and significant changes in physical examination, blood pressure, heart rate, and electrocardiogram at all concentrations of pimecrolimus throughout the study (**Table VI**). The only consistent side-effect recorded was a transient feeling of warmth occurring about 40 min after ingestion of the drug in 10 patients in the 20 mg twice daily and 30 mg twice daily cohorts, localized to the upper chest and lasting for approximately 90 min. This sensation did not occur every time after drug intake and in 75% of all cases it did not occur more often than three times during the study. Fifty-eight percent of these events occurred on the first day of drug intake, 75% of them occurred during the first 10 d of the study, and 25% between days 21 and 28 (data not shown). The sensation of warmth was not a matter of concern for the patients.

Hematology and blood chemistry revealed no clinically significant changes throughout the study (data not shown).

Special attention was paid to blood urea nitrogen and serum creatinine. There was no notable change from baseline in blood urea nitrogen, and in serum creatinine at any concentration (**Fig 4**). Blood urea nitrogen never increased more than 60% compared with baseline. Serum creatinine never exceeded 150 μmol per liter in any treatment or in the placebo group. Serum creatinine never increased more than 30% compared with baseline (**Fig 4**) except for two patients treated with pimecrolimus

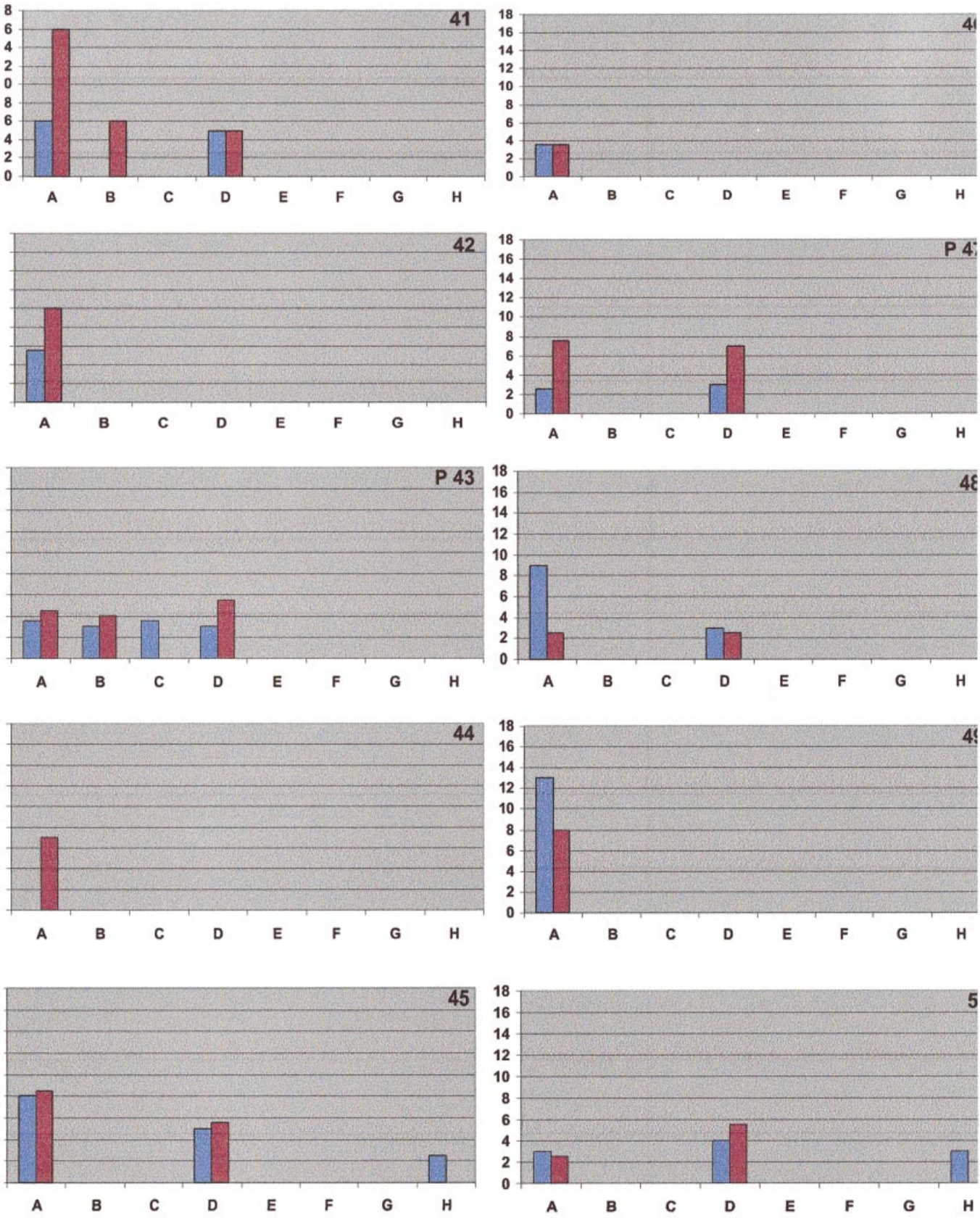


Figure 10. Skin tests for recall antigens in the 30 mg pimecrolimus twice daily patient cohort. Numbers in the right upper corner indicate patient number, patients marked with P are patients on placebo; blue bars indicate pretreatment readings, red bars post-treatment readings, A–H refer to antigens tested: (*A*) tetanus toxoid; (*B*) diphtheria toxoid; (*C*) streptococcal antigen; (*D*) tuberculin; (*E*) control; (*F*) *Candida albicans*; (*G*) *Trichophyton mentagrophytes*; (*H*) *Proteus mirabilis* antigens. The figures on the ordinate are diameters in millimeters of positive reactions. There are no consistent changes from pretreatment to post-treatment skin reactions to the recall antigens tested.

Table VII Fluorescence-activated cell sorter analysis: comparison of lymphocyte subpopulations before and after treatment, ANOVA results

Description	Estimated ratio after/before			p-value		
	Placebo	20 mg once daily	30 mg twice daily	Placebo	20 mg once daily	30 mg twice daily
Leuko (g per l)	1.05	1.10	0.97	0.719	0.282	0.715
% Lympho	1.20	1.22	1.02	0.199	0.058	0.884
Lympho (g per l)	1.26	1.34[a]	0.98	0.130	0.010	0.882
CD3 (% of leuco)	1.23	1.17	1.07	0.195	0.168	0.586
CD4 (% of leuco)	1.26	1.20	1.10	0.142	0.099	0.426
CD8 (% of leuco)	1.15	1.21	1.05	0.409	0.132	0.713
CD56 (% of leuco)	1.02	1.35[a]	0.81	0.911	0.036	0.151
CD19 (% of leuco)	1.39	1.29[a]	1.24	0.072	0.049	0.120
TCR-αβ (% of leuco)	1.19	1.20		0.391	0.094	
TCR-γδ (% of leuco)	0.95	1.17		0.808	0.181	
CD3 + 25 (% of leuco)	1.32	1.05	0.80	0.456	0.838	0.411
CD3 + HLA DR (% of leuco)	1.40	1.29	0.80	0.138	0.115	0.195
CD3 (% of lympho)	1.03	0.96	1.05	0.567	0.218	0.154
CD4 (% of lympho)	1.05	0.99	1.08[a]	0.273	0.648	0.025
CD8 (% of lympho)	0.96	0.99	1.03	0.493	0.851	0.469
CD56 (% of lympho)	0.58	1.11	0.80	0.322	0.388	0.081
CD19 (% of lympho)	1.16	1.06	1.22[a]	0.106	0.342	0.008
TCR-αβ (% of lympho)	0.95	0.98		0.115	0.298	
TCR-γδ (% of lympho)	0.75	0.96		0.338	0.783	
CD3 (g per l)	1.29	1.29[a]	1.03	0.131	0.039	0.786
CD4 (g per l)	1.32	1.32[a]	1.06	0.107	0.025	0.614
CD8 (g per l)	1.21	1.33[a]	1.02	0.274	0.025	0.899
CD56 (g per l)	1.08	1.49[a]	0.79	0.694	0.011	0.131
CD19 (g per l)	1.46	1.41[a]	1.20	0.062	0.020	0.219
TCR-αβ (g per l)	1.36	1.32[a]		0.229	0.046	
TCR-γδ (g per l)	1.08	1.38[a]		0.764	0.032	
CD3+25 (g per l)	1.34	1.28	0.84	0.469	0.386	0.577
CD3 + HLA DR (g per l)	1.41	1.42	0.79	0.162	0.050	0.193
CD25 (% of CD3)	1.07	0.90	0.75	0.857	0.678	0.282
HLA DR (% of CD3)	1.13	1.10	0.75	0.547	0.520	0.084

[a]Ratio statistically significantly different from 1.

In this table the parameter values before treatment and after 4 wk of treatment were compared for the placebo group and each of the treatment groups (20 mg once daily and 30 mg twice daily pimecrolimus). *Example:* For the lymphocytes (Lympho g per l), the ratio of the number of lymphocytes after treatment to the number of lymphocytes before treatment was estimated as 1.26 in the placebo group, i.e., the number of lymphocytes increased by 26% in the placebo group; however, this increase was not statistically significant (p-value = 0.130, > 0.05). In the 20 mg once daily group, the number of lymphocytes increased by 34%, which was statistically (p-value = 0.010). In the 30 mg twice daily group, the number of lymphocytes slightly decreased by 2% (p-value = 0.882, not statistically significant). leuco, leucocytes; lympho, lymphocytes.

20 mg twice daily who had an isolated increase of 30% at day 8 and 44% at day 14, respectively. But on days 21 and 28, and for the end of study visit serum creatinine was within the normal range in these patients. Glomerular filtration rate determined by inulin clearance and renal plasma flow determined by PAH clearance were performed at baseline, day 13, day 28, and completion of study. Glomerular filtration rate (**Fig 5**) and renal plasma flow (**Fig 6**) fluctuated more than creatinine but showed no trend for a decrease in the pimecrolimus-treated patients in comparison with the placebo-treated patients, nor with increasing doses of pimecrolimus.

Glucose tolerance tests were normal.

Histopathologically and immunopathologically pimecrolimus reverses the psoriatic phenotype to normal Histopathologic and immunopathologic examination of biopsies taken from the patients in the 20 mg once daily, 20 mg twice daily, and 30 mg twice daily cohorts were in accordance with the clinical findings and were most pronounced in the 30 mg twice daily cohort. Pimecrolimus induced an almost complete reversion of psoriatic epidermal hyperplasia and a marked reduction of the inflammatory infiltrate at 28 d of treatment (**Fig 7*a,b***). There was a significant reduction of epidermal thickness, the expression of the proliferation-associated keratin 16 (**Fig 7*a,b***) and the proliferating, i.e., Ki-67$^+$ keratinocytes (**Figs 7*c,d* and 8**). Staining patterns of involucrin and filaggrin reverted from that typical for psoriasis to near that of normal epidermis (data not shown). Keratinocyte activation (HLA-DR and intercellular adhesion molecule-1 staining) was significantly reduced or abolished (data not shown). There was a significant reduction of CD3$^+$, CD4$^+$, and CD8$^+$ dermal lymphocytes (**Fig 9*a–c***), and a significant reduction of CD3$^+$ and CD4$^+$ epidermal lymphocytes; CD8$^+$ epidermal lymphocytes were also reduced but this did not reach the level of significance (**Fig 9*a–c***); there were no significant changes in the CD1a$^+$ cell population both in the epidermis and dermis (**Fig 9*d***) and there was an involution of the proliferating papillary blood vessels and their activation markers (data not shown).

Similar albeit lesser changes were also seen in the 20 mg once daily cohort (see **Fig 9*a,b***) where no noticeable effect of pimecrolimus on psoriasis was evident clinically. Placebo-treated patients retained the full complement of the histopathologic and immunopathologic phenotype of psoriasis (data not shown).

Pimecrolimus leaves immunologic parameters unaffected Intradermal testing for delayed hypersensitivity reactions to recall antigens showed no significant changes from pretreatment to post-treatment testing for the pimecrolimus and placebo-treated patients (**Fig 10**). Flow cytometry analysis of blood lymphocyte subpopulations revealed no significant differences between treatment groups, and placebo patients (**Tables VII and VIII**). With the exception of a significant increase of the proliferation response to superantigen at a 250 ng per ml concentration in the placebo patients and in the 20 mg once daily cohort no consistent and significant changes or patterns were seen in the proliferation of

Table VIII. Fluorescence-activated cell sorter analysis: comparison of parameter changes treatment *vs* placebo, ANOVA results

	Estimated ratio treatment/placebo		p-values	
Description	20 mg once daily	30 mg twice daily	20 mg once daily	30 mg twice daily
Leuko (g per l)	1.05	0.92	0.735	0.613
% Lympho	1.01	0.84	0.935	0.342
Lympho (g per l)	1.07	0.78	0.714	0.191
CD3 (% of leuco)	0.95	0.87	0.790	0.468
CD4 (% of leuco)	0.96	0.87	0.806	0.470
CD8 (% of leuco)	1.05	0.91	0.825	0.659
CD56 (% of leuco)	1.32	0.79	0.238	0.333
CD19 (% of leuco)	0.93	0.89	0.733	0.590
TCR αβ (% of leuco)	1.01		0.971	
TCR γδ (% of leuco)	1.23		0.405	
CD3 + 25 (% of leuco)	0.80	0.60	0.622	0.280
CD3 + HLA DR (% of leuco)	0.92	0.57	0.760	0.056
CD3 (% of lympho)	0.94	1.02	0.241	0.668
CD4 (% of lympho)	0.94	1.03	0.250	0.563
CD8 (% of lympho)	1.03	1.08	0.650	0.329
CD56 (% of lympho)	1.30	0.94	0.197	0.760
CD19 (% of lympho)	0.92	1.05	0.418	0.641
TCR-αβ (% of lympho)	1.04		0.310	
TCR-γδ (% of lympho)	1.27		0.456	
CD3 (g per l)	1.00	0.80	0.998	0.286
CD4 (g per l)	1.01	0.81	0.976	0.308
CD 8 (g per l)	1.11	0.84	0.625	0.422
CD56 (g per l)	1.38	0.73	0.203	0.219
CD19 (g per l)	0.96	0.82	0.878	0.417
TCR-αβ (g per l)	0.97		0.914	
TCR-γδ (g per l)	1.28		0.402	
CD3 + 25 (g per l)	0.96	0.63	0.929	0.364
CD3 + HLA DR (g per l)	1.01	0.56	0980	0.064
CD25 (% of CD3)	0.84	0.70	0.699	0.426
HLA DR (% of CD3)	0.97	0.66	0.904	0.129

[a]Ratio statistically significantly different from 1.

In this table the changes in parameter values before and after treatment with pimecrolimus were compared with the corresponding changes in the placebo group. Leuco, leucocytes; lympho, lymphocytes.

lymphocytes (examples shown in **Table IX**); also, no consistent patterns were seen in cytokine release before and after 4 wk of treatment (examples shown in **Table X**).

Follow-up shows recurrence of psoriasis after 8 wk but no rebound The 20 patients of the 20 mg twice daily and 30 mg twice daily cohorts, four of whom had received placebo, were followed at varying intervals up to 12 mo. Twelve of the responders had a recurrence after 8 wk after termination of treatment (median 6 wk, range 4–20 wk). Recurrences were as follows: seven of 12 had a full recurrence (100% of original PASI), three of 12 a recurrence up to 80%, and two of 12, 30% of original PASI. Rebound effects (i.e., worsening of psoriasis over baseline) were not noted.

DISCUSSION

In this study we show that the ascomycin macrolactam pimecrolimus: (i) has a gene profile of broad anti-inflammatory activity without evidence of toxicity as evaluated in blood cells; (ii) exhibits excellent clinical tolerability after 4 wk of oral treatment up to the highest dose tested (30 mg twice daily); (iii) after multiple oral treatments blood concentrations reach steady state after 5–10 d and drug exposure at a steady state is linearly concentration dependent; and (iv) given in doses of 20 mg twice daily and 30 mg twice daily pimecrolimus is highly effective in a concentration-dependent manner in patients with moderate to severe plaque psoriasis.

Pimecrolimus is a selective inhibitor of inflammatory cytokines *in vitro*. By binding to macrophilin-12 it inhibits the Ca-dependent phosphatase calcineurin[1] and by blocking the transcription of early cytokines and thus downregulating, at nanomolar concentrations, both T helper 1 and 2 type cytokine synthesis (Grassberger *et al*, 1999) it inhibits T cell activation *in vitro*. Not surprisingly, pimecrolimus exhibits high anti-inflammatory activity in animal models of skin inflammation both topically and systemically (Meingassner *et al*, 1997; Neckermann *et al*, 2000). Its potency in skin inflammation models is comparable with that of dexamethasone after topical application and, when given systemically, it exceeds that of cyclosporine A by factors ranging from 2 to 4 and is equally effective or superior to tacrolimus depending on the model chosen (Meingassner *et al*, 1997; Stuetz *et al*, 2001). By contrast, in animal models of systemic immunosuppression it has only low activity when compared with cyclosporine A and tacrolimus (Stuetz *et al*, 2001). For instance, in the rat kidney transplantation model an oral dose three times higher than that of cyclosporine A and 15 times higher than that of tacrolimus is required to prevent kidney rejection and in a model of localized graft versus host reaction, pimecrolimus is less potent than cyclosporine and tacrolimus in suppressing graft versus host reactions by a factor of 8 and 66, respectively (Stuetz *et al*, 2001). In contrast to cyclosporine and tacrolimus the oral treatment of mice with pimecrolimus neither impairs the primary immune response nor decreases the lymph node weight and cellularity in allergic contact dermatitis (Meingassner *et al*, 2000). These data indicate that pimecrolimus has unique pharmacologic properties by combining high skin-selective anti-inflammatory activity with a low potential for systemic immune responses, suggesting its suitability for treatment of T cell-mediated autoreactive inflammatory reactions of the skin in humans.

In this study it has been shown that pimecrolimus is highly effective in psoriasis in a concentration-dependent manner. The impressive clinical efficacy of pimecrolimus compares favorably with that of established systemic treatments for psoriasis, such as psoralen + ultraviolet A photochemotherapy and oral retinoid–psoralen + ultraviolet A combinations (Hönigsmann *et al*, 1994), methotrexate (Linden and Weinstein, 1999), and cyclosporine A (Lebwohl *et al*, 1998). Clinical remission of psoriasis was accompanied by corresponding histopathologic and immunomorphologic reversion of the psoriatic to a normal or almost normal phenotype. Curiously, a significant reduction of $CD3^+$ and $CD4^+$ T cells was observed in the 20 mg pimecrolimus once daily cohort without clinical improvement. This may be taken to indicate that treatment for a prolonged time may result in clinical effectiveness even at lower doses of the drug.

To the best of our knowledge the gene expression analysis reported here is one of the first of its kind and the results shown are consistent with the observed clinical efficacy and the tolerability of pimecrolimus. Whereas pimecrolimus was shown to downregulate expression of genes strongly for the target pathway, inflammation, activation, proliferation, chemotaxis, and migration of leukocytes, and it did not reveal changes in gene expression that might be linked to treatment-related immunosuppression and toxicity. The predictive value of such pharmacogenomic investigations has to be confirmed but the data presented here harmonize with the observed efficacy and tolerability of the drug.

No impairment of organ function as assessed by clinical and laboratory examinations and no systemic immunosuppression was noted in this study and no viral infections were seen throughout the time patients were followed. This appears to verify the observation in animal models where pimecrolimus was shown to have high skin-selective anti-inflammatory activity and a very low potential for effecting systemic immune responses (Stuetz *et al*, 2001). There were three episodes of minor common cold. The feeling of warmth noted as the only consistent adverse effect in our patients is as yet unexplained. Although it appears to be concentration dependent it

Table IX. (*a*) proliferation of T lymphocytes: comparison of parameters before and after treatment, ANOVA results

	Estimated ratio after/before			p-value		
Description	Placebo	20 mg once daily	30 mg twice daily	Placebo	20 mg once daily	30 mg twice daily
Background	1.05	1.28	0.79	0.895	0.357	0.422
SEA/B 250 (ng per ml)	1.51[a]	1.19	0.82	0.005	0.071	0.050
SEA/B 65.5 (ng per ml)	1.53	1.55[a]	0.96	0.080	0.016	0.804
SEA/B 3.9 (ng per ml)	1.74	1.18	0.80	0.253	0.630	0.531
PHA	1.18	2.08[a]	1.00	0.738	0.049	0.994
SEA/B 250/background	1.44	0.92	1.03	0.322	0.760	0.910
SEA/B 65.5/background	1.46	1.21	1.21	0.373	0.529	0.556
SEA/B 3.9/background	1.66	0.92	1.00	0.397	0.835	0.991
PHA/background	1.12	1.62	1.26	0.842	0.250	0.594

[a]Ratio statistically significantly different from 1The parameter values before treatment and after 4 wk of treatment were compared for the placebo and treatment groups. Statistically significant increases were identified for placebo (SEA/B 250 ng per ml and the 20 mg once daily group (SEA/B 65.5 ng per ml, PHA). SEA/B: superantigen SEA, SEB.

(*b*) Proliferations of T lymphocytes: comparison of parameter changes treatment *vs* placebo, ANOVA results

	Estimated ratio treatment/placebo		p-values	
Description	20 mg once daily	30 mg twice daily	20 mg once daily	30 mg twice daily
Background	1.22	0.75	0.667	0.554
SEA/B 250 (ng per ml)	0.78	0.54[a]	0.134	0.001
SEA/B 65.5 (ng per ml)	1.01	0.62	0.974	0.120
SEA/B 3.9 (ng per ml)	0.68	0.46	0.504	0.202
PHA	1.76	0.85	0.356	0.793
SEA/B 250/background	0.64	0.72	0.326	0.466
SEA/B 65.5/background	0.83	0.83	0.712	0.718
SEA/B 3.9/background	0.55	0.61	0.418	0.502
PHA/background	1.44	1.13	0.607	0.870

[a]Ratio statistically significantly different from 1.

The changes in parameter values before and after treatment with pimecrolimus were compared with the corresponding changes in the placebo group. The only statistically significant difference was observed for SEA/B 250 ng per ml (placebo *vs* 30 mg twice daily). Note that SEA/B 250 ng per ml showed a large increase by 51% in the placebo group. SEA/B: superantigen SEA, SEB.

does not occur after every drug dose and is not associated with any other systemic or laboratory abnormality. Assessments of circulating or locally released mediators, including neuropeptides, were not performed during the heat episodes.

The efficacy in psoriasis and short-term safety of pimecrolimus are most encouraging. Issues to be dealt with in the future include: safety profile of pimecrolimus when it is administered in these and lower doses over a longer period of time; determination of the number of people who achieved greater than 90% improvement and determining if response is consistent with recurrent courses of therapy. Multicenter trials are in progress to answer these and other questions. Independent of this our studies seem to indicate that the anti-inflammatory qualities of pimecrolimus are indeed unique and support the notion that pimecrolimus may be effective for other T cell-mediated skin diseases.

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Table X. (*a*) Cytokines: comparison of parameters before and after treatment, ANOVA results

	Estimated ratio after/before			p-value		
Description		Placebo	20 mg once daily	30 mg twice daily	Placebo	20 mg once daily	30 mg twice daily
IL-2 (pg per ml) medium	1.51	1.31	0.405	0.283			
IL-2 (pg per ml) PHA + PMA	0.85	0.85		0.744	0.516		
IL-4 (pg per ml) medium	1.86	0.74	1.63	0.184	0.356	0.165	
IL-4 (pg per ml) PHA + PMA	0.92	1.45[a]	1.71[a]	0.706	0.029	0.005	
IFN-γ (pg per ml) medium	3.71[a]	0.42	0.23[a]	0.039	0.051	0.005	
IFN-γ (pg per ml) PHA + PMA	0.93	1.47	1.11	0.800	0.078	0.637	
IL-10 (pg per ml) medium	3.48[a]	0.99		0.027	0.976		
IL-10 (pg per ml) PHA + PMA	1.20	1.30[a]		0.241	0.006		
TNF-α medium	1.37	1.03		0.693	0.948		
TNF-α PHA + PMA	1.20	0.93		0.799	0.839		

[a]Ratio statistically significantly different from 1.

The parameter values before treatment and after 4 wk of treatment were compared for the placebo group and each of the treatment groups. Statistically significant increases were identified for the placebo group (IFN-γpg per ml medium, IL-10 pg per ml medium), the 20 mg once daily group (IL-4 pg per ml PHA + PMA, IL10 pg per ml PHA + PMA) and the 30 mg twice daily group (IL-4 pg per ml PHA + PMA, IFN-γpg per ml medium).

(*b*) Cytokines: comparison of parameter changes treatment *vs* placebo, ANOVA results

	Estimated ratio treatment/placebo		p-values	
Description	20 mg once daily	30 mg twice daily	20 mg once daily	30 mg twice daily
IL-2 (pg per ml) medium	0.87		0.793	
IL-2 (pg per ml) PHA + PMA	1.00		0.999	
IL-4 (pg per ml) medium	0.40	0.88	0.112	0.821
IL-4 (pg per ml) PHA + PMA	1.58	1.86[a]	0.109	0.039
IFN-γ (pg per ml) medium	0.11[a]	0.06[a]	0.008	0.002
IFN-γ (pg per ml) PHA + PMA	1.58	1.20	0.212	0.627
IL-10 (pg per ml) medium	0.29[a]		0.042	
IL-10 (pg per ml) PHA + PMA	1.09		0.609	
TNF-α medium	0.75		0.745	
TNF-α PHA + PMA	0.77		0.750	

[a]Ratio statistically significantly different from 1.

The changes in parameter values before and after treatment with pimecrolimus were compared with the corresponding changes in the placebo group. Statistically significant differences were observed for IL-4 (pg per ml) PHA + PMA (placebo *vs* 30 mg twice daily), IFN-γ(pg per ml) (placebo *vs* 20 mg once daily) and IL-10 (pg per ml) medium.

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