

Pimecrolimus Inhibits the Elicitation Phase but Does Not Suppress the Sensitization Phase in Murine Contact Hypersensitivity, in Contrast to Tacrolimus and Cyclosporine A¹

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Pimecrolimus (SDZ ASM 981, Elidel) is a nonsteroid inflammatory cytokine inhibitor specifically developed for the treatment of inflammatory skin diseases. Its effect on the elicitation and sensitization phases of oxazolone-induced contact hypersensitivity was compared with tacrolimus and cyclosporine A (CyA) in BALB/c mice using the ear swelling assay. The compounds were administered orally. Elicitation was dose-dependently inhibited by all three compounds. The minimal effective doses were 30 mg per kg (pimecrolimus, tacrolimus) and 90 mg per kg (CyA), respectively. There was no impairment of sensitization by pimecrolimus up to the highest dose tested (120 mg per kg), in contrast to CyA (60% inhibition at 60 mg per kg) and tacrolimus (71% inhibition at 30 mg per kg). Weight and cellularity

of the draining lymph nodes in mice treated with tacrolimus or CyA during sensitization were reduced. In addition, proliferation of T cells after secondary stimulation was inhibited in cell cultures from lymph nodes of mice treated with tacrolimus or CyA. Thus, in contrast to tacrolimus and CyA, pimecrolimus exerts a more selective immunomodulatory effect. It does not impair the primary immune response (sensitization phase) but effectively inhibits the secondary phase, the elicitation phase that is the clinical manifestation of contact hypersensitivity. Key words: allergic contact dermatitis/cyclosporine A/DNBS/DNFB/elicitation phase/mouse/oxazolone/pimecrolimus/sensitization phase/tacrolimus. J Invest Dermatol 121:77–80, 2003

The ascomycin macrolactam derivative pimecrolimus (SDZ ASM 981, Elidel) is a nonsteroid inflammatory cytokine inhibitor specifically developed for the treatment of inflammatory skin diseases. Clinical studies in more than 4000 patients with atopic dermatitis have proved topical pimecrolimus to be highly efficacious and safe in adults, children, and infants as young as 3 mo (Harper *et al*, 2001; Luger *et al*, 2001; Eichenfield *et al*, 2002; Kapp *et al*, 2002; Van Leent *et al*, 2002; Wahn *et al*, 2002). A phase I/II 4 wk study in patients with psoriasis revealed oral pimecrolimus to be highly efficacious and well tolerated (Rappersberger *et al*, 2002). The therapeutic potential of this compound for skin diseases has been identified in laboratory animals with experimentally induced contact hypersensitivity (allergic contact dermatitis, ACD), used as a model of Langerhans-cell-dependent T-lymphocyte-mediated skin inflammations (Meingassner *et al*, 1997). Inhibition of the elicitation phase of ACD has been used as a pharmacodynamic parameter to profile topical and oral pimecrolimus in mice, rats, and domestic pigs.

ACD develops in two phases, the clinically silent sensitization phase and the clinically apparent elicitation phase, which is the physically disturbing inflammatory phase. The sensitization phase is initiated by the epicutaneous exposure to a hapten and is characterized by antigen processing and presentation by Langerhans cells and a consecutive priming of hapten-specific T cells in the draining lymph node. In the elicitation phase, upon repeated contact with the sensitizer local release of inflammatory mediators modulates the skin microenvironment, which facilitates extravasation of inflammatory leukocytes and hapten-specific T lymphocytes amplifying a background inflammatory response to the hapten into a more vigorous hapten-specific process (Grabbe *et al*, 1996; Grabbe and Schwarz, 1998; Tsuji *et al*, 2000)

The objective of this study was to investigate whether pimecrolimus has also an effect on the sensitization phase and to compare the activity of pimecrolimus, tacrolimus, and cyclosporine A (CyA) at equal oral doses on both the elicitation and sensitization phases in the same experimental setting. Both comparators are also calcineurin inhibitors and, although originally developed as immunosuppressants for the prevention of allograft rejection, are now used in dermatology, either topically as Protopic (tacrolimus) or orally as Neoral (CyA), against inflammatory skin diseases (Koo, 1995; Ruzicka *et al*, 1999; Bornhövd *et al*, 2001).

MATERIALS AND METHODS

Test compounds Pimecrolimus (SDZ ASM 981) was synthesized at Novartis Pharma, Basle, Switzerland, and prepared as a 20% solid solution. Tacrolimus (FK 506, Prograf) and CyA (Neoral) were used as marketed products, purchased from a local pharmacy. The final doses

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Abbreviations: ACD, allergic contact dermatitis; DNBS, 2,4-dinitrobenzenesulfonic acid; DNFB, 2,4-dinitrofluorobenzene.

were adjusted by dilutions with the required amount of liquid dosing medium (vehicle) under continuous stirring immediately before use. The doses, expressed as weight of test substance (mg) per weight (kg) of test animals, were applied in volumes of 10 ml per kg body weight.

Laboratory animals Ten-week-old to 12-wk-old female BALB/cAnNCrl mice were used. The animals were supplied by Charles River, Sulzfeld, Germany, and maintained conventionally under standardized conditions ($22 \pm 1^\circ\text{C}$, $55\% \pm 5\%$ relative humidity, 10 changes of fresh air, per hour 12 h day/12 h night cycle) in Makrolon cages with sawdust bedding. Standard laboratory chow (SNIFF R/M-H diet for mice and rats, Soest, Germany) and drinking water was given *ad libitum*. In each experiment groups of six to 16 mice were used after an acclimatization period of 8–10 d. Two days before the principal investigations the animals were transferred groupwise from the stock housing to the experimental housing in type III cages (up to 10 mice per cage).

The animals were used according to an approved protocol under the license MA 4009/99 (Magistratsabteilung No. 58, Amt der Wiener Landesregierung).

Test on elicitation phase In three separate studies groups of eight mice each were sensitized on the shaved abdomen with 50 μl of oxazolone (Sigma-Aldrich, St. Louis, MO; 2% in acetone) on day 1 and challenged with 10 μl 0.5% oxazolone on the inner surface of the right ear. The unchallenged left ears served as normal controls and contact hypersensitivity reaction was evaluated from the difference in auricular weights (as a measure of inflammatory swelling) 24 h after the challenge when the response peaks. The test animals were treated twice orally (2 h before and 4 h after the challenge) with pimecrolimus, tacrolimus, or CyA at dose levels of 10, 30, and 90 mg per kg. Control animals were treated with the vehicle only.

Test on sensitization phase Groups of eight or 16 animals were sensitized with oxazolone on day 1 as described above and treated orally with the test compounds 2 h before and 4, 24, and 48 h after the exposure to oxazolone. Pimecrolimus was administered at 30, 90, and 120 mg per kg, tacrolimus at 10 and 30 mg per kg, and CyA at 30 and 60 mg per kg. Control animals were treated with the vehicles alone. Two days after the last treatment (day 5) the animals were sacrificed and the draining lymph nodes were excised, weighed, and collected in RPMI 1640 medium (GibcoBRL, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU penicillin per ml, 100 μg streptomycin per ml, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 1% minimum of nonessential amino acids (GibcoBRL). Lymph node cell suspensions were prepared by gently pressing the mechanically disrupted tissue through a sterile nylon sieve (cell strainer, 70 μm , Becton Dickinson-Falcon, Le Pont De Claix, France). The cells were washed and resuspended in RPMI medium, and cells 5–20 μm in size were counted electronically (CASY 1 TT, Schärfe System, Reutlingen, Germany). Cell viability was analyzed with a flow cytometer (FACSCalibur flow cytometer) and accompanying Cell Quest software (Becton Dickinson, San Jose, CA) after staining with 7-aminoactinomycin D (7-AAD, BD-Pharmingen, Los Angeles, CA). (The ratio of nonviable 7-AAD-positive cells was similar in all inocula (3%–4.5%).) Aliquots were used to inject naive recipient animals intravenously with 5×10^7 primed lymph node cells (Salerno *et al*, 1998). Immediately after the cell transfer, the recipients (eight per dose group) were challenged with oxazolone on the ears and evaluated for hypersensitivity response 24 h later as described in the previous section. Three independent studies were performed.

(Adoptive transfer experiments have been preferred to challenge studies in actively sensitized and treated mice in order to exclude inhibition of the elicitation phase by possible drug residuals from treatment performed at the sensitization period a short time before.)

Test on hapten-specific T cell proliferation In three studies groups of eight mice were sensitized on the shaved abdomen with 50 μl 0.5% 2,4-dinitrofluorobenzene (DNFB, Sigma-Aldrich, in acetone:olive oil, 4:1 vol/vol) on day 1 and treated orally with 90 mg per kg pimecrolimus, 10 mg per kg tacrolimus, 30 mg per kg CyA, or vehicle 2 h before and 4, 24, and 48 h after exposure to the hapten. On day 5, six draining lymph nodes were dissected and lymph node cell suspensions from each animal were prepared as described in the previous section. The cells were suspended in supplemented RPMI 1640 (4×10^6 cells per ml) and placed in triplicate in 100 μl medium into flat-bottomed wells (96-well plates). For *in vitro* hapten pulse, 100 μl 0.5 mM aqueous solution of 2,4-dinitrobenzenesulfonic acid sodium salt (DNBS, a water-soluble analog of DNFB, Sigma-Aldrich) were added to the cells (Nuriya *et al*, 2001). After incubation in a humidified atmosphere at 37°C for 3 d, hapten-specific cell proliferation was assessed with a colorimetric immunoassay (Roche

Diagnostics, Mannheim, Germany) using a 16 h 5-bromo-2'-deoxyuridine incorporation. The assay was performed according to the manufacturer's instruction. For comparison cultures without hapten pulse were used.

Statistical analyses Individual data of test and control (vehicle-treated) groups obtained from repeated studies were pooled and evaluated with one-way analysis of variance followed by Dunnett's *post hoc* test (parametric test) or by Kruskal-Wallis and Dunn's test (nonparametric) using the SigmaStat[®] program, version 2.3.

RESULTS

Pimecrolimus and tacrolimus equally inhibit the elicitation phase of ACD; both are superior to CyA Contact hypersensitivity reaction was dose-dependently and with equivalent potency and efficacy inhibited by oral treatment with pimecrolimus and tacrolimus (**Fig 1**). Treatment with 2×90 mg per kg resulted in an inhibition by 58%–63%. Doses of 2×30 mg per kg led to an inhibition by 40%, whereas doses of 2×10 mg per kg had effects below the level of statistical significance. CyA showed significant inhibition only at the highest dose tested (2×90 mg per kg).

Pimecrolimus does not interfere with the sensitization phase of ACD, in contrast to tacrolimus and CyA Contact hypersensitivity response in passively sensitized mice was not inhibited when the transferred lymph node cells were collected from mice treated orally with pimecrolimus during the sensitization phase (**Fig 2**). Even four doses of 120 mg per kg pimecrolimus (i.e., >8-fold the minimal total dose needed to inhibit the elicitation phase), given 2 h before and 4, 24, and 48 h after the hapten exposure did not result in statistically significantly different hypersensitivity responses compared to vehicle-treated mice. In contrast, treatment with tacrolimus at 4×30 mg per kg (i.e., 2-fold the minimal total dose inhibiting the elicitation phase) resulted in an inhibition by 72%. A similar effect was observed with CyA at 60 mg per kg (1.3-fold the minimal total dose needed to inhibit the elicitation phase). The impairment of the sensitization phase by tacrolimus and CyA was dose-dependent.

The interference of tacrolimus and CyA with the sensitization phase was associated with an inhibition of the hyperplasia of the draining lymph nodes (**Table I**). Hyperplasia was inhibited by 44% (weight) and 40% (cellularity) with tacrolimus at 4×30 mg per kg and by 53% (weight) and 54% (cellularity) with 60 mg per kg CyA. No effect on lymph node hyperplasia was observed in pimecrolimus-treated mice even up to the highest dose tested (4×120 mg per kg).

As inhibition of lymph node hyperplasia indicated a suppression of antigen-specific lymphocyte proliferation, additional studies on hapten-specific T cell proliferation with DNFB and its water-soluble analog DNBS were performed *ex vivo*.

Inhibition of the sensitization phase of ACD by tacrolimus is associated with impaired proliferation of antigen-specific lymph node cells *in vitro* Cultured lymph node cells from DNFB-sensitized and vehicle-treated mice exhibited a 9- or 13-fold increased proliferative response after a pulse with DNBS compared to cells from naive mice (**Table II**). The antigen-specific proliferative response was not inhibited in lymph node cells collected from mice treated with pimecrolimus at the highest dosage tested (four doses of 90 mg per kg). In contrast, response to the hapten pulse was significantly inhibited by 37% in lymph node cell cultures prepared from DNFB-sensitized mice treated with 10 mg per kg tacrolimus or by 65% in cells from mice treated with 30 mg per kg CyA.

DISCUSSION

The side by side comparison of pimecrolimus, tacrolimus, and CyA in mice showed that the two phases of ACD are differently affected by the compounds although inhibition of calcineurin

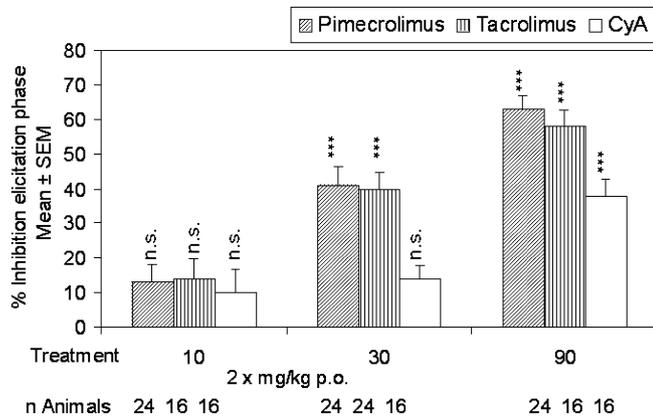


Figure 1. Pimecrolimus and tacrolimus inhibit the elicitation phase of ACD equally and more efficaciously than CyA. Treatment-related inhibition of the elicitation phase was determined in actively sensitized mice by assessment of differences in auricular weights in drug- and vehicle-treated animals. Treatment was performed 2 h before and 4 h after challenge: n.s., not significantly different ($p > 0.05$) from vehicle-treated animals (controls); *** $p < 0.001$ vs controls (differences in auricular weights in controls 24 h after challenge: 27.5 ± 4.7 mg).

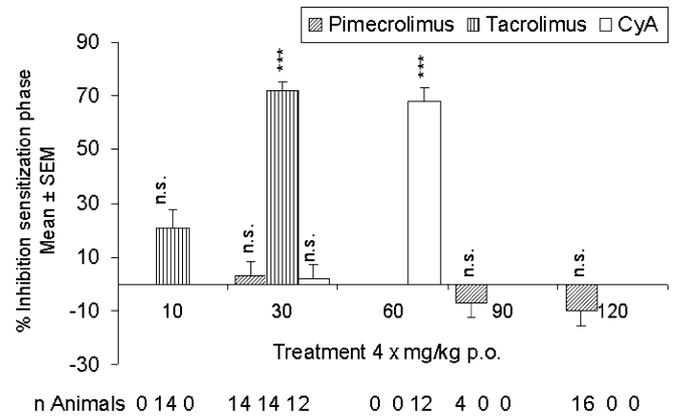


Figure 2. Pimecrolimus does not interfere with the sensitization phase of ACD in contrast to tacrolimus and CyA. Inhibition of the sensitization phase was assessed by the hypersensitivity response at challenged ears (differences in auricular weight) of mice passively sensitized with transferred lymph node cells from donors treated 2 h before and 4, 24, and 48 h after sensitization; n.s., not significantly different ($p > 0.05$) from vehicle-treated animals (controls); *** $p < 0.001$ vs controls (differences in auricular weights in controls 24 h after challenge: 19.5 ± 3.2 mg).

Table I. Effects of treatment with pimecrolimus, tacrolimus, or CyA during the induction phase of ACD on weight and cellularity (*in italics*) of draining lymph nodes

Treatment (4 × mg per kg oral)	% Inhibition of lymph node hyperplasia (weight), % Reduction in cellularity (mean ± SEM (number of animals))		
	Pimecrolimus	Tacrolimus	CyA
120	6 ± 3.5 n.s. (20) 13 ± 6.7 n.s. (12)	—	—
90	10 ± 2.5 n.s. (27) 8 ± 6.8 n.s. (12)	—	—
60	—	—	53 ± 1.7 *** (31) 54 ± 5.7 *** (16)
30	3 ± 2.0 n.s. (27) -8 ± 5.4 n.s. (12)	44 ± 1.6 *** (27) 40 ± 3.7 *** (12)	9 ± 3.2 ** (10) 17 ± 5.2 ** (8)
10	—	17 ± 2.1 *** (27) 6 ± 6.4 n.s. (12)	—

—, not tested; n.s., not significantly different ($p > 0.05$) from vehicle-treated animals (controls).
** $p < 0.01$, *** $p < 0.001$ vs controls.

Table II. Antigen-specific proliferative response of lymph node cells from DNFB-sensitized mice with/without pimecrolimus, tacrolimus, or CyA treatment

Treatment group	Proliferative response, mean ± SD, OD 450 (number of animals)	% Inhibition, mean ± SEM
Pimecrolimus ^I	0.60 ± 0.15 (8)	$8 \pm 11.1\%$ n.s.
Tacrolimus ^I	0.41 ± 0.10 (8)	$37 \pm 7.5\%$ ***
CyA ^{II}	0.16 ± 0.12 (8)	65 ± 4.7 ***
Vehicle I-treated sensitized controls	0.65 ± 0.26 (8)	—
Vehicle II-treated sensitized controls	0.46 ± 0.23 (8)	—
Non-sensitized controls	0.05 ± 0.03 (16)	—

—, not tested; n.s. not statistically significantly different ($p > 0.05$) from DNFB-sensitized and vehicle-treated animals (controls); I, II, corresponding vehicle.
*** $p < 0.001$ vs controls.

activity and thereby thus inhibition of gene transcription is the common molecular mechanism of action. Pimecrolimus and tacrolimus inhibited the inflammatory response of the elicitation phase with similar potencies and efficacies and were superior in activities to CyA when equal doses, expressed as mg per kg, were used. In comparison with oral doses used in patients with psoriasis (Ho *et al*, 1999; Anonymous, 1996; Rappersberger *et al*, 2002) the minimal effective doses in mice are high. It has to be considered, however, that the murine dose equivalent factor of these compounds is higher than the commonly calculated factor of 12.5 (Van Mierth, 1989) and that therapeutic approaches and treatment schedules are different. On a molar basis, CyA (MW 1202) was under-dosed by approximately one-third in relation to pimecrolimus and tacrolimus, which were tested in almost equimolar amounts (MW 811 and MW 822, respectively). The same doses affecting the elicitation phase had been used to study the influence of the compounds on the induction phase with the difference that the animals were treated with four doses distributed over three consecutive days to ensure drug exposure during the sensitization period. Doses of 30 or 90 mg per kg pimecrolimus, which inhibited the elicitation phase equally to tacrolimus and better than CyA (Fig 1), did not interfere with the sensitization phase. Even doses of 120 mg per kg pimecrolimus had no statistically significant effects (Fig 2). In contrast, treatment with tacrolimus at 30 mg per kg or CyA at 60 mg per kg resulted in a pronounced inhibition of sensitization. This effect was associated with an inhibition of the hyperplasia of the draining lymph nodes in the treated animals (Table I). Therefore, twice as many tacrolimus- and CyA-treated cell donors than pimecrolimus-treated donors were needed for the transfer studies. A comparison at the 90 mg per kg level was impossible because the lymph nodes of tacrolimus-treated animals were highly atrophic and animals treated with 4 × 90 mg per kg CyA died or had to be killed due to severe toxic signs ahead of schedule. As expected, inhibition of sensitization was accompanied by impaired proliferation of antigen-specific lymph node cells *ex vivo* (Table II). This assay proved to be very sensitive as the compounds inhibited hapten-specific T cell proliferation at doses that only marginally affected lymph node weight and cellularity and did not significantly inhibit the inflammatory ear response in passively sensitized mice.

The differential effects of pimecrolimus, tacrolimus, and CyA on the sensitization phase *in vivo* are surprising and need further studies to elucidate these pharmacodynamic differences. In fact, previous studies showed that CyA is more than 10-fold less potent than pimecrolimus and tacrolimus at the T cell level *in vitro* (Tocci *et al*, 1989; Grassberger *et al*, 1999; Kalthoff *et al*, 2002). Furthermore, a recent side by side comparison of pimecrolimus and tacrolimus addressing the inhibition of the stimulated expression of interleukin-2 (IL-2), IL-3, IL-8, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor α in the human T cell line Jurkat showed that pimecrolimus was nearly as potent as tacrolimus at both the mRNA level and the protein level.² Although this appears to be in conflict with our findings, it may be explained by a distinct distribution and bioavailability of the active drugs in skin, subcutaneous lymphoid compartment, and draining lymph nodes. Indeed, the tissue distribution of the two compounds differed in rats treated orally with pimecrolimus or tacrolimus.³ The concentration of pimecrolimus in skin was higher than that of tacrolimus but the concentration in lymph nodes was lower. As another possibility, the compounds may differentially affect antigen-presenting cells or T cells as the relevant

immunologic targets in the cascade from the first hapten contact in the skin to the priming of antigen-reactive T cells in the draining lymph nodes. In this context, it is interesting that human Langerhans cells exposed *ex vivo* to 10⁻⁸ mol per liter tacrolimus showed reduced expression of major histocompatibility complex class I and II antigens and costimulatory molecules, such as CD40 and CD80, and exhibited a severely reduced T cell stimulatory ability in a skin mixed lymphocyte reaction (Panhans-Gross *et al*, 2001).

These data demonstrate that pimecrolimus has a more specific pharmacologic profile than CyA and tacrolimus, indicating that pimecrolimus might have a lower potential for side-effects. This, however, has to be confirmed by a head to head comparison in clinical studies.

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