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Effects of the phosphodiesterase 4 inhibitor RPR 73401 in a model of immunological inflammation

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Abstract

The study was performed to investigate effects of the phosphodiesterase 4 inhibitor RPR 73401 [*N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamid] on an allergic skin reaction. To simulate an immunological inflammation, BALB/c mice were sensitized to dinitrochlorobenzene or toluenediisocyanate. At first, the abdominal skin was shaved and 50 μl Freund's adjuvant were injected intracutaneously once. Then, the horny layer was removed by adhesive tape stripping and 100 μl 0.5% dinitrochlorobenzene or 5% toluenediisocyanate were administered on the epidermis for 4 days. After repeated local treatment of the ear skin with 20 μl 3% RPR 73401 or intraperitoneal administration of 1 and 5 mg/kg RPR 73401, 20 μl 1% dinitrochlorobenzene or 0.5% toluenediisocyanate were given topically as a challenge. The vehicle controls showed a high increase in ear thickness over 48 h after challenge, whereas RPR 73401 administered on either route reduced this increase significantly. Nevertheless after topical administration, RPR 73401 had a longer lasting effect. These and other results may point to an indication for RPR 73401 in immunological dermatitis.

Keywords: Phosphodiesterase 4 inhibitor; RPR 73401; Allergic dermatitis; Ear swelling test, mouse; Interleukin-4

1. Introduction

Immunological inflammatory cells with their mediators initiate and promote the complex chronic progress of allergic skin diseases (Hanifin, 1991; Werfel and Kapp, 1998; Krasteva et al., 1999), but also epithelial cells (keratinocytes) have more functions than just a barrier (Tenor et al., 1995; Chujor et al., 1998; Kaiserlian and Etchart, 1999). As an important intracellular regulatory substance, the second messenger cyclic-3′,5′-adenosine monophosphate (cAMP) suppresses the inflammatory reaction by activation of proteinkinase A especially in lymphocytes (Palfreyman and Souness, 1996). Cell specifically distributed phosphodiesterase isoenzymes catalyse the breakdown of cAMP (Nicholson et al., 1991). Methylxanthines obviously inhibit the phosphodiesterases unspecifically (Rabe et al., 1995), but only weakly (Palfreyman and Souness, 1996; Nuhn, 1997). Especially, theophylline is used even today as a bronchodilatory and anti-inflammatory agent in bronchial asthma (Rabe et al., 1995; Wettengel, 1998).

Currently, the mammalian phosphodiesterase isoenzymes are divided into 9 gene families (formerly I–IX) that are pharmacologically differentiated by their special substrate (also cGMP) and selective inhibitors. They include isogene subtypes and within these isoform splice variants (Dousa, 1999). The development of novel phosphodiesterase inhibitors is of major interest (Dent and Rabe, 1996), especially those of the rolipram-sensitive cAMP-specific phosphodiesterase 4 isoenzyme (Palfreyman, 1995; Polymeropoulos and Höfgen, 1997) that dominates in allergically relevant inflammatory cells (Torphy and Undem, 1991; Nuhn, 1997). Phosphodiesterase 4 inhibitors have been shown to suppress inflammatory reactions in nose and lung (Palfreyman and Souness, 1996; Marx et al., 1997).

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In leukocytes of patients with atopic dermatitis, especially children, a very high phosphodiesterase 4 activity was found (Grewe et al., 1982; Butler et al., 1983; Cooper et al., 1985). Therefore, this disease could be an indication for phosphodiesterase 4 inhibitors (Chan and Hanifin, 1993; Crocker and Townley, 1999). Pathophysiologically, there probably is an excessive stimulation of antigene presenting cells (AP-cells) in the skin (Langerhans cells) and a surplus activation of TH_2-cells (Hanifin and Chan, 1996). Interleukin-1 from AP-cells and keratinocytes increases the interleukin-secretion by activated T-lymphocytes. Interleukin-4 from TH_2-cells, e.g., induces the release of B-cells and production of immunoglobulin E (IgE). Interleukin-10 from TH_2-cells and AP-cells effects an inhibition of TH_1-cell expansion (Hanifin and Chan, 1996).

The use of selective phosphodiesterase 4 inhibitors synthesized so far, e.g., rolipram (Beavo and Reifsnyder, 1990; Dent et al., 1991), partly is excluded due to gastrointestinal and central nervous side effects (Barnette et al., 1996; Duplantier and Biggers, 1996; Poppe and Szelenyi, 1999). Current investigations are supposed to show effects of novel phosphodiesterase 4 inhibitors in the skin (Teixeira et al., 1994; Hanifin et al., 1996). Particular mechanisms of inflammatory processes and the influence of phosphodiesterase 4 inhibitors on these are studied in cell cultures (Donath et al., 1999; Heer et al., 1999, Küsters et al., 1999). A few phosphodiesterase 4 inhibitors are already in phase I of clinical testing, most of them are still in the preclinical development (Silvestre et al., 1998).

RPR 73401 [RP 73401, Piclamilast, *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamid] is one of the newer inhibitors of the rolipram-sensitive cAMP-specific phosphodiesterase 4. The inhibitory concentration for 50% (IC_{50}) of the phosphodiesterase 4 in vitro is 0.41 nmol/l RPR 73401 (Szelenyi, 2000, personal communication) and no other pharmacological effects regarding inhibition of immune responses were observed in screening tests with this concentration. RPR 73401 is 3–14-fold more potent than rolipram in increasing cAMP accumulation (Souness et al., 1995) and inhibiting several eosinophil functions (Palfreyman, 1995). The described experiments were performed to investigate effects of RPR 73401 on an allergic inflammatory skin reaction. RPR 73401 was administered systemically in one group and topically in another to study, if — a sufficient therapeutic effect provided — possible systemic side effects can be avoided on this way. To simulate an immunological inflammation, the mouse ear swelling test with mice that had been sensitized according to Gad et al. (1986) to dinitrochlorobenzene or toluenediisocyanate was used. These contact allergens have different phenotypes of response due to the underlying pathophysiological changes. Dinitrochlorobenzene is increasing the production and secretion of interleukin-2 in TH_1-cells (Dearman et al., 1996) for delayed allergic reaction (cellular, type IV), whereas phosphodiesterase 4 inhibitors and the positive control cyclosporin A inhibit this cytokine. On the other hand, toluenediisocyanate is increasing the production and secretion of interleukin-4 in TH_2-cells (Dearman et al., 1996) for immediate allergic reaction (IgE, type I), which is identical to atopic dermatitis. Here, systemically given dexamethasone — not cyclosporin A — finally served as a positive control, since only this was supposed to potently — though unspecifically — reduce ear swelling of toluenediisocyanate-challenged mice.

2. Materials and methods

2.1. Animal model

Female BALB/c-mice were obtained from Charles River, Sulzfeld (Germany) at the age of 8 weeks (20 g body weight). All animals were healthy, housed in groups of four to six mice per cage at 22°C with a 12-h light/dark cycle. Water and a standard diet (Altromin, Lage/Lippe, Germany) were available ad libitum. The animal experiment had been registered by Bezirksregierung, Hannover, Germany (Az. 509i-42502-98A839).

After settling in for 1 week, the abdominal skin was shaved and depilated (Veet®, Reckitt & Colman, Hamburg, Germany), and 50 μl Freund's adjuvant (Sigma-Aldrich Chemie, Deisenhofen, Germany) were injected intracutaneously once. Then, the horny layers of the abdominal skin were stripped off ten times with adhesive tapes (Tesafilm®, Beiersdorf, Hamburg, Germany). For active sensitization, 100 μl 0.5% dinitrochlorobenzene (Sigma-Aldrich Chemie) in acetone/dimethylsulfoxide (DMSO) (1:1) (Merck, Darmstadt, Germany) or 100 μl 5% toluenediisocyanate (Sigma-Aldrich Chemie) in acetone (Merck) each were administered on the local epidermis for 4 consecutive days (Fig. 1).

Ten days later, the allergic reaction was challenged by administration of 10 μl 1% dinitrochlorobenzene in acetone/DMSO (1:1) or 10 μl 0.5% toluenediisocyanate in acetone each onto the inner and outer surface of the left ears to examine the sensitization status, whereas the right ears were used as *individual controls* (administration of the vehicle with the same volume). Before as well as 24 and 48 h afterwards, the ear thickness was measured with a cutimeter (model 7309, Mitutoyo, Neuss, Germany). The

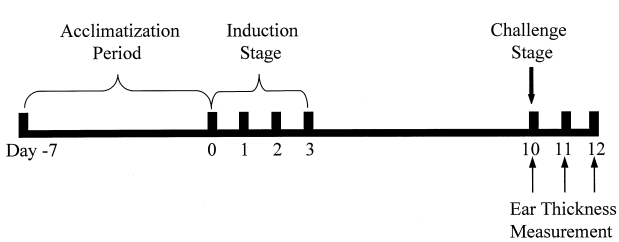

Fig. 1. Time schedule of mouse sensitization and allergy control.

percent swelling was calculated by comparison of the values before challenge with 24 and 48 h after challenge. The individual swelling of the right ears at these two time points was substracted from the left ear ones to distinguish allergen-independent changes in ear thickness. Animals that had a mean swelling difference of less than 20% 24 and 48 h after challenge compared to the earlier assessed individual basal value (ca. 210 μm) were sorted out as being not sensitized. The other mice were equally distributed to the treatment groups ($n = 6$) in the order of their swelling intensity, so that each group contained animals which had responded to different extents. They were rested for further experiments until the ear thickness had reached almost a normal level after 10 days and no residues of the allergen on the ears had to be feared.

2.2. Experiments

2.2.1. Dinitrochlorobenzene-sensitive mice

Twice in 24 h, RPR 73401 (Batch 2613a, Arzneimittelwerk, Dresden, Germany) was given intraperitoneally as systemic treatment [1 mg/kg, 10 ml/kg, vehicle containing 10% PEG200 (Merck) and 0.45% hydroxyethyl cellulose (Caesar & Loretz, Hilden, Germany)]. For topical administration, the skin of the left and right ears were treated with 20 μl 3% RPR 73401 in acetone/DMSO (1:1) at the same time points. Negative controls were given the vehicle intraperitoneally and topically, respectively. A positive control group was injected with cyclosporin A (Sandoz, Nürnberg, Germany) suspended in Miglyol 812 (Caesar & Loretz) subcutaneously (30 mg/kg, 5 ml/kg). Two hours after the second treatment, 20 μl 1% dinitrochlorobenzene were administered to the right ear skin, which had not been used for a challenge so far. On the left ears, 20 μl acetone/DMSO (1:1) were administered as the *individual control*. Before as well as 24 and 48 h after this challenge, the ear thickness was measured again and the percent change of the left ears was substracted from the right ones to assess the swelling difference.

After a sufficient wash out phase (5 days), the treatment with topical RPR 73401 and systemic cyclosporin A as well as the challenge were repeated once more. The mice were sacrificed 24 h after dinitrochlorobenzene administration by cervical dislocation. The heparinized (Roth, Karlsruhe, Germany) blood (20 IU/ml) of each group was pooled, plasma was gained by centrifugation (model 5403, Eppendorf, Hamburg, Germany) with 2000 ***g*** for 10 min at 4°C and freezed at −80°C. One half of the used ears was fixed in 4% formaldehyde (Fluka, Deisenhofen, Germany) for histological section, stained with haematoxylin-eosin with respect to epidermal thickness and granulocytes accumulation. These parameters were measured in 10 high powered fields with 40 times magnification. The remaining tissue was shock-frozen in liquid nitrogen and stored at −80°C until homogenization (20000 rpm, 40 s, 4°C; Ultra Turrax®, Janke & Kunkel, Staufen, Germany) with 0.05% Tween20 (Sigma-Aldrich Chemie) and 1% bovine serum albumin (Sigma-Aldrich Chemie) in phosphate buffered saline (Merck). After centrifugation, interleukin-4 was determined in the supernatant and in the plasma by ELISA (monoclonal rat anti-mouse interleukin-4 and biotinylated polyclonal goat anti-mouse interleukin-4; DuoSeT®, Genzyme, Rüsselsheim, Germany) to distinguish between local and systemic effects of the used drugs, possibly in dependence on their route of administration. For quantification of the homogenized tissue, also the amount of protein in the supernatant was measured by the method of Lowry et al. (1951).

2.2.2. Toluenediisocyanate-sensitive mice

As in the described systemic administration experiments with dinitrochlorobenzene-sensitive mice, the animals were treated intraperitoneally with RPR 73401, its vehicle or subcutaneously with 30 mg/kg cyclosporin A twice in 24 h. Instead of 1 mg/kg RPR 73401, according to Poppe and Szelenyi (1999), the dose was increased to 5 mg/kg for the indirect inhibition of the toluenediisocyanate-effect needed due to the different TH-cells and interleukins involved with phosphodiesterase 4 inhibitors and this allergen. Two hours afterwards, the animals were challenged with 20 μl 0.5% toluenediisocyanate in acetone on the right ears.

After unsatisfactory results, the experiment was modified by administration of 5 mg/kg RPR 73401 intraperitoneally or 20 μl 3% RPR 73401 on the skin of the left and right ear four times every 4 h. A control group was treated intraperitoneally and topically with the vehicles. As positive control, another group was given 1 mg/kg dexamethasone intraperitoneally 15 and 2 h before challenge. One hour after the first treatment with RPR 73401 or its vehicles, the right ears were challenged with the antigene. Because toluenediisocyanate induces earlier allergic reac-

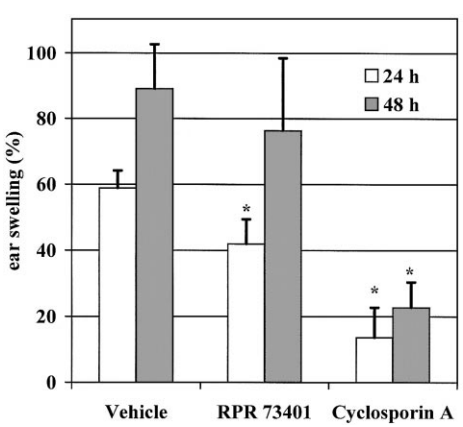

Fig. 2. Ear swelling 24 and 48 h from local dinitrochlorobenzene challenge after systemic pretreatment with 1 mg/kg RPR 73401, its vehicle or 30 mg/kg cyclosporin A. Data given as mean (% of basal value [ca. 210 μm] − 100) + S.D. of six mice per group. * Significant difference to vehicle controls ($P < 0.01$, *U*-test).

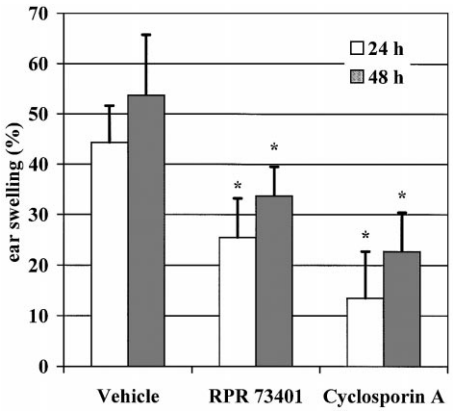

Fig. 3. Ear swelling 24 and 48 h from local dinitrochlorobenzene challenge after pretreatment with 3% RPR 73401 topically, its vehicle or 30 mg/kg cyclosporin A systemically. Data given as mean (% of basal value [ca. 210 μm] − 100) + S.D. of six mice per group. * Significant difference to vehicle controls ($P < 0.01$, *U*-test).

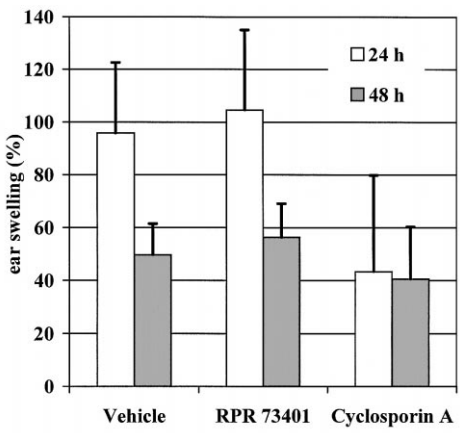

Fig. 4. Ear swelling 24 and 48 h from local toluenediisocyanate challenge after systemic pretreatment with 5 mg/kg RPR 73401, its vehicle or 30 mg/kg cyclosporin A. Data given as mean (% of basal value [ca. 210 μm] − 100) + S.D. of six mice per group.

tions than dinitrochlorobenzene (Dearman et al., 1996), as could be interpreted from the results of the preceding experiment, also the ear swelling 6 h after challenge was now assessed.

2.2.3. Statistical evaluation

The different treatment groups were checked for significant different effects in the model with the Mann–Whitney *U*-test. Since the vehicle group was compared to two or three other groups, the α-error was adjusted with the Bonferoni correction.

3. Results

3.1. Dinitrochlorobenzene-sensitive mice

3.1.1. Systemic treatment

In dinitrochlorobenzene-sensitive mice, the vehicle control with systemic treatment showed a mean increase of 59% and 89% in ear thickness 24 and 48 h after challenge, respectively (Fig. 2). The swelling at both time points was nearly prevented by subcutaneous injection of cyclosporin A, whereas RPR 73401 given intraperitoneally had a significant effect only 24 h after challenge.

3.1.2. Topical treatment

The vehicle control with local treatment showed a mean increase of 44% and 54% in ear thickness 24 and 48 h after challenge, respectively (Fig. 3). By topical administration of RPR 73401, this swelling was significantly reduced not only 24 h, but also 48 h after challenge.

Twenty four hours after local dinitrochlorobenzene challenge, vehicle pretreated mice showed an increased epidermal thickness and granulocytes accumulation (eosinophil and other) in the ear skin compared to the unchallenged group (Table 1). The increase of both parameters was reduced by topically administered RPR 73401, though this was not significant. Cyclosporin A (subcutaneously) held back the epidermal thickness and granulocytes score almost on the basal level. Regarding the granulocytes score, biologically determined high variations have to be taken into consideration.

Also, the interleukin-4 content in the ear skin was significantly higher in the vehicle group 24 h after dinitrochlorobenzene exposure than in the unchallenged one. The increased value in the ear skin was not reduced by local RPR 73401, but completely by systemically administered cyclosporin A. In the plasma of these two groups, the interleukin-4 level seemed much lower than in the plasma of the vehicle treated animals, but it has to be remembered

Table 1
Histological and biochemical parameters
Results 24 h after local dinitrochlorobenzene challenge and pretreatment with 3% RPR 73401, its vehicle or 30 mg/kg cyclosporin A systemically. Data given as mean ± S.D. of six mice per group. Significant difference to vehicle controls: a = $P < 0.05$, b = $P < 0.01$ (*U*-test). n.m. = not measured

	Not challenged controls	Vehicle controls	RPR 73401 topically	Cyclosporin A
Epidermal thickness (μm)	16.5 ± 3.1	30.0 ± 9.1	25.3 ± 9.1	19.3 ± 5.3
Granulocytes score in dermis	no cells	medium	low	low
Interleukin-4 in ear skin (pg/mg protein)	24 ± 5[a]	38 ± 13	38 ± 8	23 ± 3[b]
Interleukin-4 in pooled plasma (pg/ml)	n.m.	117	35	27

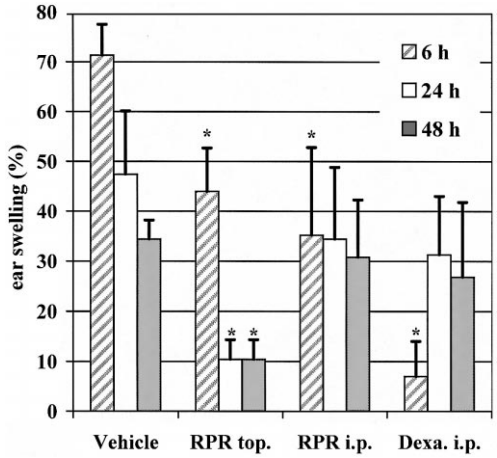

Fig. 5. Ear swelling 6, 24 and 48 h from local toluenediisocyanate challenge during multiple treatment with 3% RPR 73401 topically, 5 mg/kg RPR 73401 intraperitoneally, its vehicles or 1 mg/kg dexamethasone intraperitoneally. Data given as mean (% of basal value [ca. 210 μm]−100)+S.D. of six mice per group. * Significant difference to vehicle controls ($P < 0.01$, *U*-test).

that no individual data and standard deviations are available, since due to low volume the plasma of each group had to be pooled.

3.2. Toluenediisocyanate-sensitive mice

In toluenediisocyanate-sensitive mice, the vehicle control showed a mean increase of 96% and 50% in ear thickness 24 and 48 h after challenge, respectively (Fig. 4). Only during the first 24 h, the swelling was reduced by cyclosporin A, but neither the 48 h increase of ear thickness with cyclosporin A nor one of both time points with RPR 73401 differed clearly from the vehicle group.

In the modified experiment, the vehicle control showed a mean increase of 71%, 47% and 34% in ear thickness 6, 24 and 48 h after challenge, respectively (Fig. 5). This swelling was nearly inhibited by dexamethasone after 6 h. At this time, also RPR 73401 had a significant effect on the local and intraperitoneal route. During the following 42 h, the topically administered RPR 73401 reduced the ear thickness almost to normal values, whereas the other treatment groups found their level at about 30% swelling.

4. Discussion

4.1. Animal model

The described study was performed to investigate effects of systemically or topically administered RPR 73401 on an allergic skin reaction in vivo. Therefore, the mouse ear swelling test was established by successfully sensitizing mice to dinitrochlorobenzene or toluenediisocyanate (Gad et al., 1986). This was shown by the mean increase of 44% to 96% in ear thickness 24 and 48 h after dinitrochlorobenzene or toluenediisocyanate challenge in the vehicle control groups. The used concentrations of dinitrochlorobenzene and toluenediisocyanate for induction and challenge were lower than the primarily irritating ones (Gad et al., 1986), thus the secondary local reaction was specifically immunological. This was controlled by exposing naive nonsensitized mice to the allergens showing no unexpected irritation signs. The measured histological and biochemical parameters of inflammation and allergy, respectively (Table 1), showed significant effects of the dinitrochlorobenzene challenge in the vehicle control group compared to the unchallenged basal values. Moreover, also in the positive control groups, subcutaneous cyclosporin A or intraperitoneal dexamethasone avoided the increase of ear swelling or of other parameters significantly as expected relative to the vehicle groups (Figs. 2–5, Table 1).

In both experiments with dinitrochlorobenzene, the skin thickening of the vehicle groups increased between 24 and 48 h after challenge (Figs. 2 and 3). According to Gad et al. (1986), the expression of the secondary response to toluenediisocyanate application started quickly within the first 6 h (Fig. 5) once primary sensitization in the mouse was achieved by induction, but faded rapidly during the following 42 h (Figs. 4 and 5). This difference probably was also caused by the varying ability of dinitrochlorobenzene and toluenediisocyanate to permeate the skin, apart from the different type of allergic reaction onset. On the one hand, the horny layer may act as a nonpenetrateable barrier when the volatile vehicles of the antigens vaporize quickly after administration (Gad et al., 1986), leaving behind the allergens on the epidermis as cristalls, or the horny layers may serve as a reservoir for these agents. On the other hand, acetone destroys the barrier of the horny layers and DMSO is an enhancer of skin permeation, not only for the allergens, but also for therein dissolved topically applied drugs like RPR 73401. These aspects need further evaluation.

4.2. Experiments

4.2.1. Dinitrochlorobenzene-sensitive mice

By the systemic or topical administration of RPR 73401, the increase in ear thickness after dinitrochlorobenzene challenge was reduced. On the topical route, RPR 73401 had a longer lasting effect (Fig. 3). The missing reduction of ear swelling 48 h after challenge in the experiment with intraperitoneal treatment (Fig. 2) may be caused by a quick metabolisation and excretion of RPR 73401 on the systemic route. According to Griswold et al. (1998), this could also reflect a poor penetration of RPR 73401 from the vasculature into the cutaneous compartment. Nevertheless, clinically, in case of an allergic reaction, the initial reduction of the sensitivity is more important.

The apparant results on the histological level (Table 1) fit well to the measurements with the cutimeter (Fig. 3),

though physiologically the epidermis makes only 15% of the whole ear thickness and edema formation as well as leukocyte infiltration in the dermal compartment during skin inflammation has to be differentiated from hyperproliferation of the epidermis (e.g., keratinocytes). Therefore, the constancy of epidermal thickness by treatment of challenged ears was not expected to differ significantly from the vehicle controls. The reduction of the interleukin-4 concentration in the plasma after dinitrochlorobenzene challenge by topically administered RPR 73401 and cyclosporin A systemically (Table 1) perhaps was an indirect effect, since phosphodiesterase 4 inhibitors (Palfreyman and Souness, 1996) as well as cyclosporin A (Olyaei et al., 1998) primarily inhibit interleukin-2 production, whereas dinitrochlorobenzene increases the production and secretion of this cytokine (Dearman et al., 1996). Thus, in the ear skin, only cyclosporin A acted via the systemic cascade, but RPR 73401 given topically had no inhibitory effect on the production and release of interleukin-4. To control the observed potential of the active principles in reduction of ear swelling, also toluenediisocyanate that directly induces interleukin-4 (Dearman et al., 1996) like in atopic dermatitis was used for challenge in specifically sensitized mice.

4.2.2. Toluenediisocyanate-sensitive mice

Using an identical study design as for the dinitrochlorobenzene-sensitive mice with systemic administration of the same test substances, just with cyclosporin A and only during the first 24 h, the increase of ear thickness was reduced (Fig. 4). This sounds explainable as neither the positive control nor RPR 73401 directly act on the interleukin-4 production. Nevertheless, there could be a pharmacokinetic reason for the missing inhibition of ear swelling, as this was already observed after systemic administration of RPR 73401 in dinitrochlorobenzene-sensitive mice 48 h after challenge (Fig. 2). For this reason, the experiment was repeated with multiple treatments using topically and systemically administered RPR 73401 and dexamethasone as an unspecific positive control.

In the second experiment with toluenediisocyanate-sensitive mice, the inhibitory effect of dexamethasone was significant 6 h after challenge (Fig. 5). As in the studies with dinitrochlorobenzene challenge, topically administered RPR 73401 reduced the ear swelling more than the intraperitoneal injection. Since the phosphodiesterase 4 inhibitor was constantly given on both routes from 1 h before challenge until 11 h afterwards, the results of systemic administration may have been more a question of reduced distribution into a peripheral compartment like the skin as described by Griswold et al. (1998) than quick metabolisation and excretion in the central compartment.

It has to be questioned why the ear swelling in the vehicle control group with topical treatment (Figs. 3 and 5) is significantly lower than after the systemic one (Figs. 2 and 4) — a phenomenon that was observed with toluenediisocyanate-sensitive mice ($P < 0.02$, *U*-test) as well as with dinitrochlorobenzene-sensitive mice ($P < 0.01$, *U*-test). Maybe the topical DMSO that made 50% of the vehicle for RPR 73401 had an antiinflammatory effect itself, but the dinitrochlorobenzene was administered in the same vehicle. This is investigated in the future.

In conclusion, the mouse ear swelling test as an in vivo model for immunological skin inflammation was successfully established, as nearly every animal was sensitive to dinitrochlorobenzene or toluenediisocyanate after induction and the positive controls showed the expected effect. In both groups of administration, RPR 73401 reduced the increase in ear thickness, but had a longer lasting antiallergic potential in the epidermis on the topical route than on the systemic one. These and other results point to an indication for RPR 73401 in immunological skin diseases, especially with direct application to the epidermis that may diminish systemic side effects (Palfreyman and Souness, 1996). More studies will be performed to supplement the observations made.

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