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Effects of the phosphodiesterase 4 inhibitors SB 207499 and AWD 12-281 on the inflammatory reaction in a model of allergic dermatitis

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Abstract

The inhibitors of the phosphodiesterase 4, SB 207499 (cilomilast, *c*-4-cyano-4-(3-cyclopentyloxy-4-methoxy-phenyl)-*r*-L-cyclohexane carboxylic acid) and AWD 12-281 (*N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide) were tested in a model of allergic dermatitis in mice. To obtain an allergic dermatitis, BALB/c mice were sensitized to toluene-2,4-diisocyanate. The allergic reaction was challenged by topical administration of toluene-2,4-diisocyanate onto the mice ears. Before challenge, two groups of mice were treated topically (ear skin) with SB 207499 or AWD 12-281. There was a significant ear swelling in toluene-2,4-diisocyanate-challenged mice ears 4, 8, 16, 24 and 48 h after challenge. SB 207499 and AWD 12-281 inhibited this swelling significantly 8, 16, 24 and 48 h after the challenge. For biochemical parameters and histology, ears were sampled from mice sacrificed 4, 8 and 16 h after the challenge. In homogenized tissue, SB 207499 and AWD 12-281 inhibited significantly the secretion of interleukin 1β induced by toluene-2,4-diisocyanate 4 and 8 h after challenge. The cell influx (granulocytes) observed in the toluene-2,4-diisocyanate-challenged mice 8 and 16 h after challenge was nearly abolished by AWD 12-281 and SB 204799.

Keywords: Allergy; Dermatitis; Phosphodiesterase 4 inhibitor; AWD 12-281; SB 207499; Toluenediisocyanate

1. Introduction

Several studies indicate a beneficial role of phosphodiesterase 4 inhibitors in allergic diseases like asthma (Schmidt et al., 2000; Giembycz, 2000), allergic rhinitis (Marx et al., 1997) and allergic dermatitis (Hanifin et al., 1996; Ehinger et al., 2000). The phosphodiesterase 4 inhibitors block enzymes responsible for the breakdown of adenosine 3′,5′-cyclic monophosphate (cAMP) in cells. To date, 11 phosphodiesterase isoenzyme gene families have been identified (Giembycz, 2000). These isoenzymes differ in their cellular distribution and biochemical function. In leukocytes of patients with atopic dermatitis, particularly in children, a high phosphodiesterase 4 activity was found (Butler et al., 1983; Cooper et al., 1985). Additionally, phosphodiesterase 4 appears to be the predominant isoenzyme in various inflammatory cells, such as monocytes and monocyte-derived macrophages (Gantner et al., 1997), B lymphocytes (Cooper et al., 1985) and eosinophils (Dent et al., 1994). Phosphodiesterase 4 is also expressed in keratinocytes. Therefore, these cells represent potential pharmacologic targets for the control of inflammatory disorders in the skin using phosphodiesterase 4 inhibitors (Chujor et al., 1998).

SB 207499 (cilomilast, *c*-4-cyano-4-(3-cyclopentyloxy-4-methoxy-phenyl)-*r*-L-cyclohexane carboxylic acid) is a second generation highly selective phosphodiesterase 4 inhibitor with an improved therapeutic index (Torphy et al., 1997). In clinical trials, the compound is currently evaluated for the treatment of asthma (Griswold et al., 1998; Giembycz, 2000) and chronic obstructive pulmonary disease. Particularly, the experiments concerning chronic obstructive pulmonary disease have demonstrated a clinically significant increase in lung function (Giembycz, 2001). The phosphodiesterase 4 inhibitor AWD 12-281 (*N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide) was successfully tested in a model of allergic bronchoconstriction. It significantly reduced the bronchospasmogenic effect of an allergen in passively sensitized human airways (Schmidt et al., 2000). AWD 12-281 inhibits the release of inflammatory mediators

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in antigen-stimulated human cells from nasal polyps such as GM-CSF (granulocyte-macrophage colony-stimulating factor), TNF-α (tumor necrosis factor α) and histamine (Kuesters et al., 1999). After inhaled administration in domestic pigs, AWD 12-281 inhibited lung neutrophilia at 4 and 6 h after lipopolysaccharide exposure in a dose-dependent manner (Poppe et al., 2001). Additionally, AWD 12-281 inhibited the degranulation of human eosinophils in vitro (Ezeamuzie, 2001).

The described animal experiments were performed in order to study effects of topically administered SB 207499 and AWD 12-281 on an allergic inflammatory skin reaction. To simulate an immunological inflammation, the already established (Ehinger et al., 2000) *mouse ear swelling test* with mice sensitized according to Gad et al. (1986) to toluene-2,4-diisocyanate was used. Depending on the sensitization regime, the contact allergen toluene-2,4-diisocyanate induces an immunoglobulin (Ig)E-independent (short exposure) or IgE-dependent (long exposure) allergic dermatitis (Scheerens et al., 1999). The sensitization regime performed in this study corresponds to the IgE-dependent long exposure.

2. Materials and methods

2.1. Sensitization procedure

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 and were housed in groups of 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 with Veet® (Reckitt & Colman, Hamburg, Germany), and 50 μl Freund's adjuvant (Sigma-Aldrich Chemie, Deisenhofen, Germany) were injected intracutaneously once. Subsequently, horny layers of the abdominal skin were stripped off 10 times with adhesive tapes (Tesafilm® Beiersdorf, Hamburg, Germany). For active sensitization, 100 μl of 5% toluene-2,4-diisocyanate (Sigma-Aldrich Chemie) in acetone were administered to the stripped epidermis on four consecutive days.

About 16 days later, the allergic reaction was challenged by administration of 10 μl 0.5% toluene-2,4-diisocyanate in acetone (Merck, Darmstadt, Germany) on both 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 after challenge, the ear thickness was measured with a cutimeter (model 7309, Mitutoyo, Neuss, Germany). The swelling was calculated by comparison of the values before challenge with values obtained 24 and 48 h after challenge. The individual swelling of the right ear at these two time points was substracted from that of the left ear in order 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. 230 μm) were excluded as being not sensitized. The other mice were equally distributed to the treatment groups according to their swelling intensity, so that each group contained animals which had responded to varying extents. They were rested for the experiments until the ear thickness had reached almost a normal level after 16 days and residues of the allergen on the ears were no longer to be feared.

2.2. Experiments

The mice were divided into four groups.

- One group of mice, which were not sensitized and challenged, served as an untreated control.
- A second group was challenged topically by administration of 20 μl 0.5% toluene-2,4-diisocyanate in acetone to both ears.
- The third group was treated with 20 μl AWD 12-281 (600 μg in acetone/dimethyl sulfoxide (DMSO) 9:1) to both ears 2 h before toluene-2,4-diisocyanate challenge.
- The fourth group was treated with 20 μl SB 207499 (600 μg in acetone/DMSO 9:1) to both ears 2 h before toluene-2,4-diisocyanate challenge.

AWD 12-281 and SB 207499 were obtained from ASTA Medica (Dresden, Germany). To exclude effects by the vehicle (acetone/DMSO), the second group (toluene-2,4-diisocyanate) was treated also with the vehicle 2 h before the challenge. Ear thickness was measured 4, 8 and 16 h after the toluene-2,4-diisocyanate challenge. The mice ($n = 6$ each group) were sacrificed by cervical dislocation 4, 8 and 16 h after the toluene-2,4-diisocyanate challenge and the ears were collected.

One further experiment was carried out to obtain information about the influence of AWD 12-281 and SB 207499 on the late allergic response. Therefore, the ear thickness ($n = 6$ each group) was measured also 24 and 48 h after challenge. As the IC_{50} for phosphodiesterase 4 is about 10 times lower for AWD 12-281 (10 nM) compared to SB 207499 (117 nM), one additional group was treated with 0.3% AWD 12-281 (60 μg in acetone/DMSO 9:1).

One part of the ear tissue collected 8 and 16 h after the challenge was fixed in 4% formaldehyde (Fluka, Deisenhofen, Germany) and embedded in paraffin blocks for histological section and stained with haematoxylin–eosin with respect to dermal thickness and granulocyte accumulation. An additional staining (Luna) was used to differentiate between eosinophil and neutrophil granulocytes. These

parameters were measured in 10 fields at 40 times magnification. The remaining tissue was shock-frozen in liquid nitrogen immediately after sampling and stored at −80 °C until homogenization.

For the determination of biochemical parameters, the mice ears were homogenized under liquid nitrogen. The homogenates were taken in 200 μl RPMI 1640 medium (Biochrom, Berlin, Germany) and Pefabloc® (1 mmol, Boehringer Mannheim, Germany) was added and the probes were mixed intensively. After centrifugation (10,000 × *g*, 10 min, 4 °C), the supernatant was collected and the protein content was determined (Biorad®, Munich, Germany). The samples were frozen at −80 °C until the cytokines were determined. Interleukin 1β and interleukin 4 were measured in the samples by enzyme-linked immunosorbant assays (R&D Systems, Wiesbaden, Germany) using commercially available kits according to manufacturers' instructions.

2.3. Statistical evaluation

The treatment groups were compared for significantly different effects in the model using the Mann–Whitney test (*U*-test). Since the toluene-2,4-diisocyanate-treated group was compared to three other groups, the α-error was adjusted with the Bonferroni correction.

3. Results

3.1. Ear swelling

There was a mean ear swelling of 75% 24 h after the first challenge. At the early time points (4, 8 and 16 h), when samples were taken for cytokine measurement, the ear swelling was 11%, 17% and 16%. Topically administered SB 207499 as well as AWD 12-281 inhibited the toluene-2,4-diisocyanate-induced swelling significantly 8 and 16 h after toluene-2,4-diisocyanate challenge (Fig. 1). The inhibitory effect of AWD 12-281 and SB 207499 is still obvious 24 and 48 h after toluene-2,4-diisocyanate challenge (Fig. 2). The additional concentration (0.3%) chosen for AWD 12-281 resulted in a diminished response compared to application of 3% AWD 12-281 (Fig. 2).

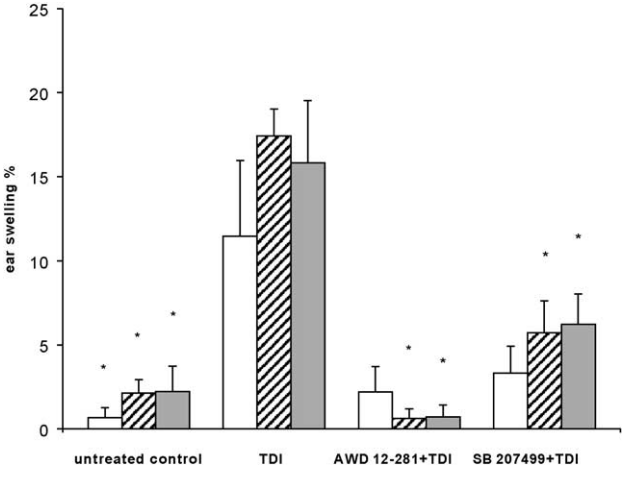

Fig. 1. Ear swelling 4 h (white bars), 8 h (hatched bars) and 16 h (grey bars) after toluene-2,4-diisocyanate challenge. There is a significant increase of the ear swelling in toluene-2,4-diisocyanate-treated mice compared to untreated controls. AWD 12-281 as well as SB 207499 inhibited the swelling (significantly 8 and 16 h after toluene-2,4-diisocyanate challenge). * $P < 0.05$ in comparison with toluene-2,4-diisocyanate-treated animals ($n = 6$ each group).

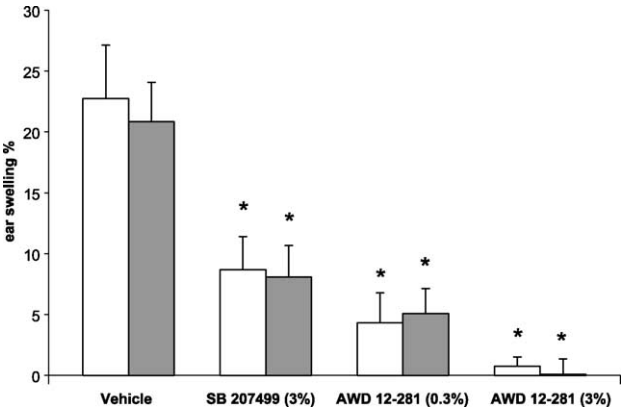

Fig. 2. Ear swelling 24 h (white bars) and 48 h (grey bars) after toluene-2,4-diisocyanate challenge (mean ± s.e. mean). AWD 12-281 (0.3% and 3%) as well as SB 207499 (3%) inhibited the toluene-2,4-diisocyanate-induced swelling significantly. * $P < 0.05$ compared to toluene-2,4-diisocyanate-treated animals ($n = 6$ each group).

3.2. Histological examination

The histological examination of the toluene-2,4-diisocyanate-challenged mice ears after 8 and 16 h shows a distinct edema. The influx of inflammatory cells accompanied by an enrichment of granulocytes in vessels is visible 8 h after the challenge. The dermis is infiltrated with granulocytes 16 h after challenge (Table 1). By an additional staining for eosinophil granulocytes, a ratio of 1:5 (eosinophils/neutrophils) was determined. AWD 12-281 as well as SB 207499 inhibited these inflammatory/allergic reactions.

Table 1
Mean values (± s.e. mean) of interleukin 4 (pg/200 μg protein) concentration and histological examination of cell influx in mice ears

	Time after challenge (h)	Untreated		Toluene-2,4-diisocyanate	
		Control	Vehicle	AWD 12-281	SB 207499
Interleukin 4	4	10 ± 1	7 ± 2	6 ± 2	8 ± 1.9
	8	10 ± 2[a]	15 ± 2	13 ± 1	9 ± 1[a]
	16	7 ± 3	13 ± 2	15 ± 4	15 ± 5
Granulocyte score in the dermis	8	−	++	−/+	+
	16	−	+++	−/+	+

[a] $P < 0.05$ compared to toluene-2,4-diisocyanate treated animals ($n = 6$).

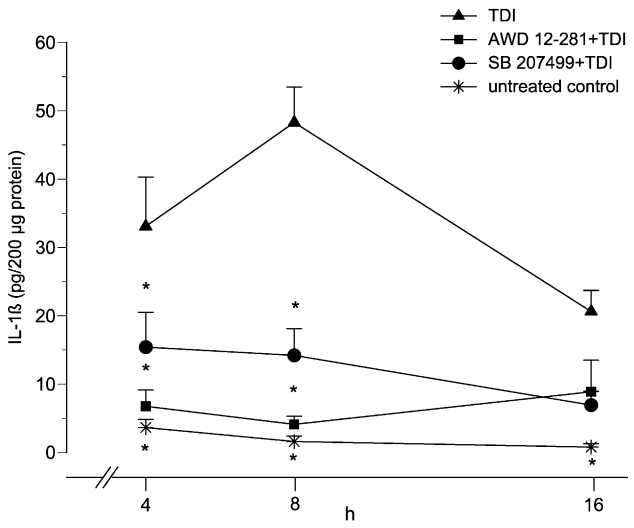

Fig. 3. Mean values (± s.e. mean) of the interleukin 1β concentration in mice ears. Toluene-2,4-diisocyanate leads to a significant elevation of interleukin 1β in mice ears. AWD 12-281 as well as SB 207499 inhibited this elevation (significantly 4 and 8 h after challenge). * $P < 0.05$ compared to toluene-2,4-diisocyanate-treated animals ($n = 6$ each group).

3.3. Inflammatory mediators in the mouse skin

There was a significant increase of the interleukin 1β concentration in the ear tissue 4, 8 and 16 h after challenge in the toluene-2,4-diisocyanate-treated group. AWD 12-281 and SB 207499 inhibited the increase of interleukin 1β concentration significantly 4 and 8 h after toluene-2,4-diisocyanate challenge (Fig. 3).

About 8 h after toluene-2,4-diisocyanate challenge, the interleukin 4 concentration was slightly, but significantly increased in the toluene-2,4-diisocyanate-treated group (Table 1), whereas SB 207499 reduced the interleukin 4 concentration to control levels. A significant increase in the toluene-2,4-diisocyanate group was not obvious 4 h after challenge and only a tendency was apparent 16 h after challenge. At the later time point, SB 20749 as well as AWD 12-281 showed no influence on the interleukin 4 concentration (Table 1).

4. Discussion

The described study was performed in order to compare the effect of two phosphodiesterase 4 inhibitors on the toluene-2,4-diisocyanate-induced mouse ear swelling. As we were interested in the early mechanisms of action of toluene-2,4-diisocyanate and the influence of the phosphodiesterase 4 inhibitors, we examined cytokine concentrations 4, 8 and 16 h after the toluene-2,4-diisocyanate challenge, which was used as a model of allergic dermatitis. As a functional parameter, the ear swelling was measured. Although there was only a slight increase of ear thickness at these time points, there is a significant inhibition of the ear swelling after topical treatment with AWD 12-281 and SB 207499. Studies on sensitization protocols for toluene-2,4-diisocyanate indicate that the magnitude of the response depends on the length of the sensitization and the interval between sensitization and challenge (Scheerens et al., 1999, own unpublished data). In this study, the interval between the sensitization and the experiment was lengthened compared to a former study (Ehinger et al., 2000). This might be the reason for the diminished response. As demonstrated in former studies (Karol et al., 1994; Scheerens et al., 1999), toluene-2,4-diisocyanate is capable of inducing different immunological reactions. The early increase in ear thickness (4 h) is considered to be IgE-mediated (type I), whereas the late response (16 h and later) represents a delayed type of hypersensitivity reaction (type IV; Tominaga et al., 1985). In order to investigate whether the phosphodiesterase 4 inhibitors influence this late response lastingly, we examined the effect of AWD 12-281 and SB 207499 in an additional experiment using the same sensitization procedure and measuring the ear swelling 24 and 48 h after the toluene-2,4-diisocyanate challenge (Fig. 2). Even 48 h after a single topical treatment with the phosphodiesterase 4 inhibitors, there is a significant inhibition of the toluene-2,4-diisocyanate-induced ear swelling. It is noteworthy that AWD 12-281 (3%) induces nearly a 100% inhibition of the ear swelling 24 and 48 h after the treatment, whereas the mean inhibition induced by SB 207499 is approximately 60%. The effect of 0.3% AWD 12-281 is diminished compared to topical administration of a 3% solution. This lower dose takes the different IC_{50}s for phosphodiesterase 4 into account. As the inhibition of the ear swelling by 0.3% AWD 12-281 is comparable to the inhibition by 3% SB 207499, there is obviously a correspondence to the IC_{50} found in vitro (IC_{50} for AWD 12-281: 10 nM compared to SB 207499: 117 nM; Griswold et al., 1998; Schmidt et al., 2000).

The stronger inhibition of the ear swelling by AWD 12-281 (3%) compared to SB 207499 (3%), which represents a more effective inhibition of edema formation and cell influx, is also obvious 8 and 16 h after the toluene-2,4-diisocyanate challenge (Fig. 1). These results are confirmed by the histological sections (Table 1).

The elevation of the proinflammatory cytokine interleukin 1β after toluene-2,4-diisocyanate challenge is consistent with the findings of Maestrelli et al. (1995) in the airway mucosa of subjects with asthma induced by toluene-2,4-diisocyanate. The distinct increase of the interleukin 1β concentration in mice ears after toluene-2,4-diisocyanate challenge may be explained by the influx of inflammatory cells and by the fact that the epidermis itself is a vast reservoir of sequestered (precurser) interleukin 1 (McKenzie and Sauder, 1990). By comparison of mRNA signals, it could be demonstrated that only contact sensitizers upregulate interleukin 1β mRNA in the skin of BALB/c mice, whereas irritants such as sodium lauryl sulfate failed to

induce an up-regulation (Enk and Katz, 1992). As interleukin 1β is an early signal in the cytokine cascade (Enk and Katz, 1992), it is not surprising that the elevation attenuates 16 h after the challenge (Fig. 3). The observed inhibition of the interleukin 1β secretion by phosphodiesterase 4 inhibitors herewith is also described for stimulated monocytes (Verghese et al., 1995). Additionally, this inhibitory effect is confirmed in attenuated plasma levels of interleukin 1β by selective phosphodiesterase 4 inhibitors in an in vivo model of profound endotoxaemia (Tofovic et al., 2000).

As an interleukin 4 overproduction is apparent in allergic diseases such as atopic dermatitis (acute affected skin lesions) (Hanifin et al., 1996; Spergel et al., 1999), we were interested in the influence of toluene-2,4-diisocyanate on interleukin 4 production in mouse skin. Only 8 h after the toluene-2,4-diisocyanate challenge, there is a significant elevation of interleukin 4 after toluene-2,4-diisocyanate treatment. However, the tendency to elevation is still evident 16 h after the challenge. Bearing in mind that the sources for interleukin 4 in the skin are limited, as keratinocytes and Langerhans cells do not produce this cytokine (Shreedhar et al., 1998; Morita et al., 2001), it is of interest that an effect is registered at all. Mast cells (Harvima et al., 1994; Dastych et al., 1999) and the influx of Th_2 cells (Shreedhar et al., 1998) are obviously the source of interleukin 4 in the skin.

The ability of SB 207499 to inhibit interleukin 4 in vivo has also been demonstrated in a model of chronic oxazolone-induced contact sensitivity (Griswold et al., 1998). The fact that there is no modulatory effect by AWD 12-281 may indicate a different mode of action. As far as the inhibition of the ear swelling and the histological section is concerned, AWD 12-281 seems to be more potent in abolishing the allergic/inflammatory symptoms. However, this is not confirmed by the interleukin 1β and interleukin 4 results in this experiment. To gain information on the important endpoints in this model, further studies screening all potential mediators responsible for the toluene-2,4-diisocyanate action in this model at various time points are under way.

As reported by Ezeamuzie (2001), AWD 12-281 shows a superior inhibitory action in vitro compared to SB 207499. In contrast to SB 207499, AWD 12-281 was able to inhibit the degranulation of human eosinophils in vitro. Additionally, AWD 12-281 inhibited an allergen-induced bronchoconstriction in passively sensitized human airways, whereas other phosphodiesterase 4 inhibitors like RPR 73401 (*N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamid) and Rolipram failed to do so (Schmidt et al., 2000).

The results of our study suggest that AWD 12-281 as well as SB 207499 may be potent modulators of allergic responses of the skin. In particular, the topical administration to the skin may, on one hand, diminish systemic side effects (Ehinger et al., 2000) and, on the other hand, this route of application guarantees a longer lasting effect, possibly due to a depot in the stratum corneum. Further studies are needed in order to investigate a long-lasting effect of phosphodiesterase 4 inhibitors in chronic allergic dermatitis.

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