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Characterization of a model of tracheal plasma extravasation in passively sensitized rats using anti-allergic and anti-inflammatory drugs by oral and intratracheal route

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ABSTRACT

The aim of the following study was to characterize a passive systemic anaphylaxis rat model of dinitrophenyl (DNP)-induced plasma extravasation in the trachea to determine if the model is appropriate for the evaluation of new drugs targeting airway mast cells by oral and intratracheal (i.t.) route. To this purpose we have used fluticasone and a range of anti-allergic drugs including compounds either active on mast cell activation, such as cromoglycate and the Syk inhibitor R406, or active on mast cell mediators, such as cetirizine and montelukast. To further characterize the model, the effect of fluticasone, cromoglycate and R406 on rat tracheal mast cell degranulation was also assessed histologically. DNP-induced tracheal plasma extravasation was inhibited by cromoglycate (i.v. and i.t.) and R406 (p.o.), but not by fluticasone (i.t.), cetirizine or montelukast (p.o.). Cromoglycate and R406 also showed inhibition of tracheal mast cell degranulation, whereas fluticasone was inactive. These results suggest that the DNP-induced tracheal plasma extravasation model constitutes a useful animal model for the evaluation, by oral and i.t. route, of new anti-allergic drugs intended to target airway mast cells.

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1. Introduction

Mast cells are tissue-resident cells, mainly found in connective tissues and mucosal surfaces, where they play an important role in host defense and repair [1]. Despite these physiological roles, mast cells have long been recognized as the key effector cells in the initiation and development of IgE-mediated allergic diseases [2]. Mast cells express the high affinity IgE receptor (FcεRI) and upon receptor engagement by an allergen-specific IgE, these cells produce and release inflammatory mediators. In asthmatic patients mast cells localize in the airway smooth muscle, the airway mucous glands and the bronchial epithelium [3] where they constitute a key element in disordered airway function in asthma [4]. The search of novel targets to modulate mast cell function has led to the

identification of Syk kinase as a potential new therapeutic strategy in allergic asthma [5] and one Syk inhibitor is in phase I by inhaled route for asthma treatment [6].

Animal models provide an important link between *in vitro* activity and *in vivo* functionality that assist in translating pre-clinical data into the human disease setting. Although there is no animal model that fully reproduces all characteristics of human asthma, a mechanistic model to assess functional effects *in vivo* can provide key insights in drug discovery establishing pharmacokinetic and pharmacodynamic (PK/PD) relationships. Passive cutaneous anaphylaxis (PCA) is a well established and validated model of skin allergic inflammation. However, PCA is not a suitable model for testing drugs by inhaled route. Mast cells are highly heterogeneous and exist as two major subtypes, mast cells containing tryptase (MC_T) and mast cells containing tryptase and chymase (MC_{TC}). In the airways MC_T is the most common subtype whereas in the skin, MC_{TC} predominates [7,8]. Mast cells from different sites exhibit different abilities to respond to pharmacological inhibitors or to different stimuli [1]. Due to this heterogeneity, the use of a model that involves mast cells from the airways is advisable when the drugs to be evaluated are intended to target airway mast cells.

The model of passive systemic immunization with anti-dinitrophenyl (DNP) IgE antibodies and subsequent DNP

Abbreviations: PK/PD, pharmacokinetic/pharmacodynamic; PCA, passive cutaneous anaphylaxis; DNP, dinitrophenyl; CysLT₁, cysteinyl leukotriene receptor 1; MC_T, mast cells containing tryptase; MC_{TC}, mast cells containing tryptase and chymase.

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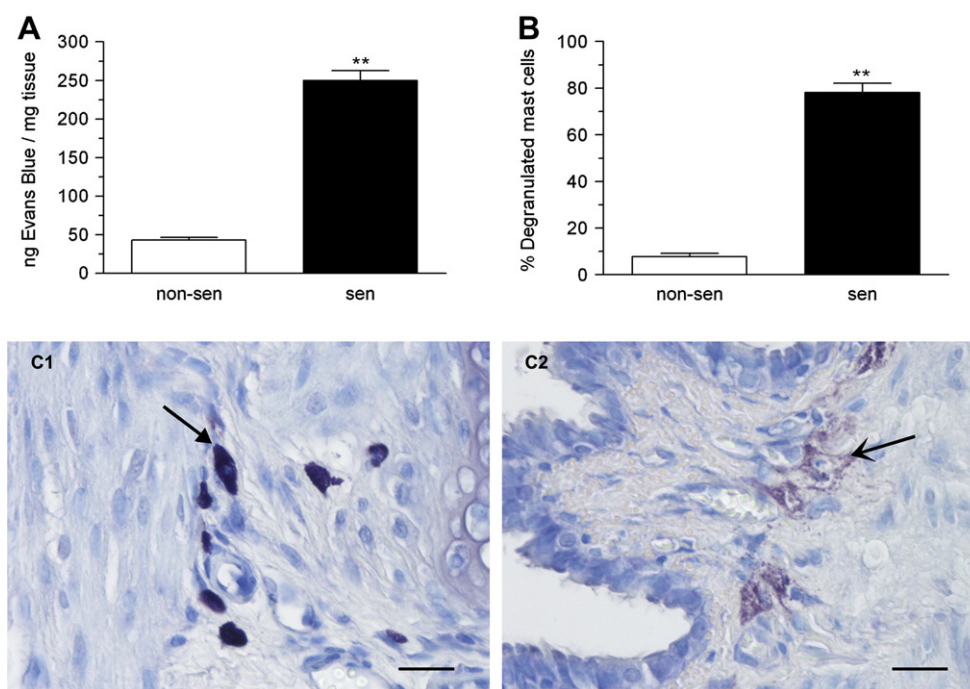


Fig. 1. DNP-induced tracheal plasma extravasation model. Animals were passively sensitized with IgE anti-DNP and challenged with DNP 24 h later. Animals were euthanized 5 min post challenge and tracheas were collected for extravasated Evans blue measurement (A) or quantification of mast cell degranulation (B and C). (C) Representative images of toluidine blue-stained tracheal sections of non-IgE sensitized rats (C1) with presence of intact mast cells (closed arrows) and IgE sensitized rats (C2) with presence of degranulated mast cells (open arrows). Non-sen: Non-treated non-IgE sensitized rats; sen: Non-treated IgE sensitized rats. Data are reported as mean $\pm$ SEM; $n = 6$. **, $p < 0.01$ compared with non-IgE sensitized group (Dunnett's test). Scale bars = 25 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

challenge, previously introduced by several authors [9,10], showed an increase in tracheal plasma extravasation that is a hallmark of acute allergic reaction. Using this model it has been described that the Syk inhibitor Bay 61-3606 by oral route inhibits DNP-induced plasma extravasation in the main bronchus of systemically sensitized rats [11]. However, relatively few studies have been conducted with other pharmacological modulators and none to our knowledge through inhaled or i.t. administration. The aim of this study was to further characterize the rat passive systemic anaphylaxis model of DNP-induced tracheal plasma extravasation using the anti-inflammatory drug fluticasone and a range of anti-allergic drugs that target mast cells, such as cromoglycate and the Syk inhibitor R406 [12] or mast cell mediators, such as cetirizine and montelukast. Since it is generally believed that plasma extravasation is dependent on IgE-bearing mast cell degranulation we also examined the ability of fluticasone, cromoglycate and R406 to inhibit tracheal mast cell degranulation. By using this approach, we aimed to determine if the model is appropriate for screening compounds targeting mast cells and if it is suitable for drug delivery by i.t. route.

2. Material and methods

2.1. Animals

Male Wistar outbred rats (6–7 weeks old; body weight, 200–225 g) raised under specific pathogen-free conditions, were obtained from Charles River Laboratories (France). They were allowed to acclimatize for 5 days and were housed in groups of 5 or 6 animals in polycarbonate cages at 22 ± 2 °C and $55 \pm 10\%$ relative humidity, with 15 air changes/h and a 12-h light–dark cycle. Rats were fed with a standard diet for rats (Harlan Teklad 2014, Madison; WI) and filtered water was available *ad libitum*. Care and use of

animals were undertaken in compliance with the European Community Directive 86/609/CEE for the use of laboratory animals and with the Autonomous Catalan law (Decret 214/1997). Experimental procedures were approved by the Almirall Ethics Committee.

2.2. Reagents

Fluticasone propionate, montelukast and R406 were synthesized by the Department of Medicinal Chemistry (Almirall SA, Barcelona, Spain); cetirizine were obtained from Dr. Reddy's Laboratories Ltd. (Hyderabad, India); disodium cromoglycate (cromolyn sodium salt), albumin-dinitrophenyl, mouse monoclonal anti-DNP antibody (clone SPE-7), Dulbecco's phosphate buffered saline (D-PBS) and Tween® 80 were purchased from Sigma–Aldrich (Madrid, Spain); formamide and formalin were purchased from Panreac Química (Castellar del Vallès, Spain); toluidine blue and methylcellulose were obtained from Scharlau (Barcelona, Spain); Evans blue, propylene glycol and polyethylene glycol 400 were purchased from Merck (Madrid, Spain); Dolethal was obtained from Vétoquinol (Alcobendas, Spain); 0.9% saline solution was obtained from Braun (Rubí, Spain); d-Alpha-tocopheryl polyethylene glycol 1000 succinate was purchased from Quimigranel (Barcelona, Spain) and isoflurane was obtained from Baxter (Valencia, Spain).

2.3. DNP-induced tracheal plasma extravasation model

Rats were anaesthetized with isoflurane (4% in oxygen) and passively sensitized by i.v. injection of 20 μ g monoclonal anti-DNP IgE antibodies in saline. Animals from the non-sensitized group received an i.v. injection of saline. Twenty four hours post sensitization, systemic plasma extravasation was induced. Sensitized and non-sensitized animals were anaesthetized with isoflurane (4% in

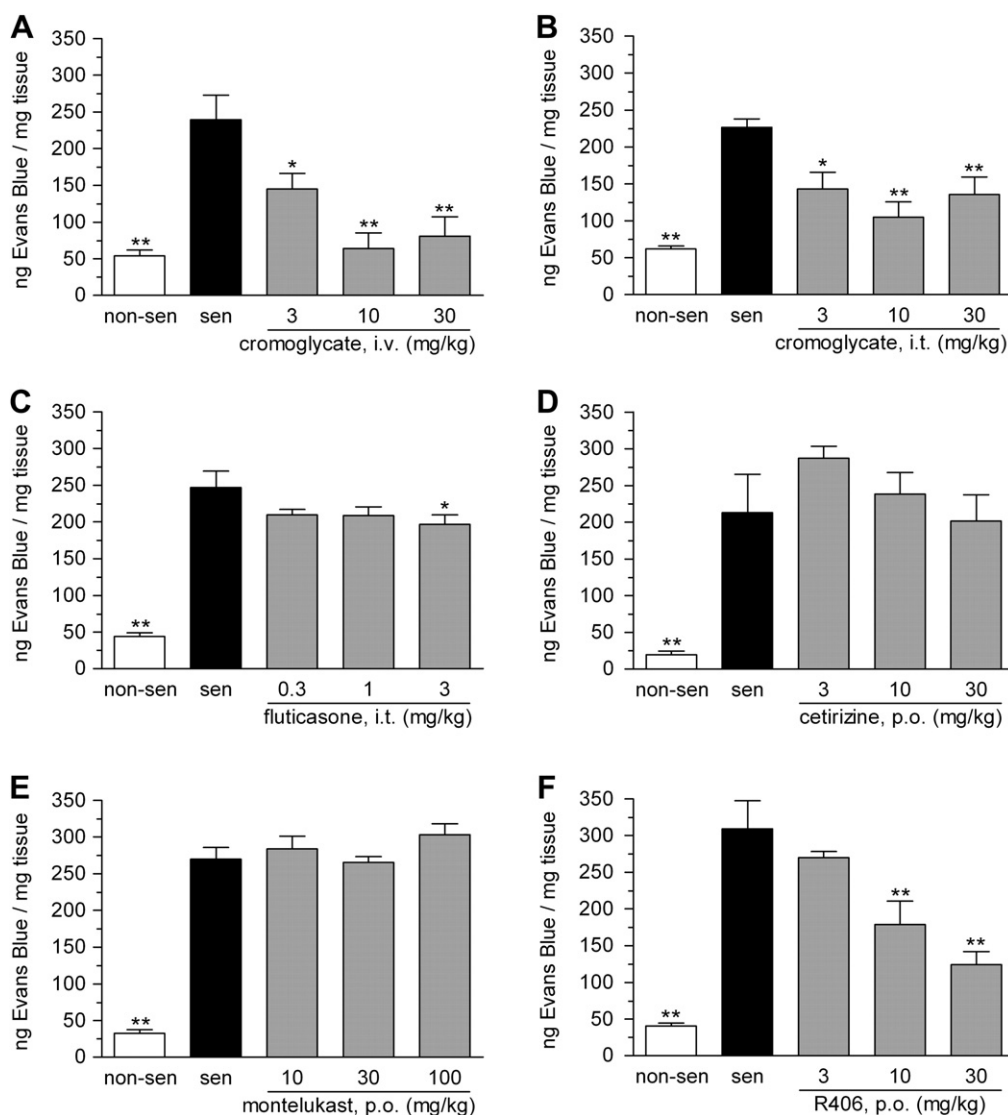


Fig. 2. Effect of (A) cromoglycate i.v., (B) cromoglycate i.t., (C) cetirizine, (D) montelukast, (E) fluticasone and (F) R406 on plasma extravasation in the trachea of sensitized rats. Animals were passively sensitized with IgE anti-DNP and challenged with DNP 24 h later. Animals were euthanized 5 min post challenge and tracheas were collected for extravasated Evans blue measurement. Cromoglycate was administered 5 min prior to DNP challenge by i.v. or i.t. route. Fluticasone was administered by i.t. route 1 h before DNP challenge. Cetirizine, montelukast and R406 were orally administered 1 h before DNP challenge. Non-sen: Non-treated non-IgE sensitized rats; sen: Non-treated IgE sensitized rats. Data are reported as mean $\pm$ S.E.M.; $n = 6$; *, $p < 0.05$, **, $p < 0.01$ compared with non-treated IgE sensitized group (Dunnett's test).

oxygen) and administered 10 μ g DNP along with 5 mg of Evans blue dye in saline by i.v. route. Five min post DNP challenge rats were euthanized with pentobarbital (Dolethal 0.4 g/kg i.p.) and tracheas were collected. Adjacent tissues were removed, tracheas were cleaned intraluminally with saline, dried and weighted for standardization. For the quantification of plasma extravasation, tracheas were submerged in 2 ml formamide and placed at 60 °C overnight. Extravasated Evans blue was quantified spectrophotometrically at 620 nm.

2.4. DNP-induced tracheal mast cell degranulation

Tracheas from both non-sensitized and passively sensitized DNP-challenged rats were removed and fixed in 10% formalin (pH 7.4) for 24 h. Each trachea was then divided into 3 segments, upper, middle and lower, embedded in paraffin and 3- μ m cross-sections were stained with 0.1% toluidine blue to evaluate the integrity of mast cells. Tracheal mast cell degranulation was quantified at all

three levels by two independent investigators in a blinded manner. Briefly, total number of mast cells, intact and degranulated, was counted at the *trachealis* muscle region, including those within the submucosa, smooth muscle and adventitia, and the percentage of degranulated mast cells was subsequently determined.

2.5. Drug administration

Disodium cromoglycate was dissolved in distilled water and administered by i.v. or i.t. route. Cetirizine and montelukast were suspended in 0.25% methylcellulose, 0.1% Tween® 80 in water and administered orally. R406 was prepared in 60% polyethylene glycol 400, 5% propylene glycol and 35% d- α -tocopheryl polyethylene glycol 1000 succinate (adjusted pH 5) as described previously [12] and administered orally. Fluticasone propionate was prepared in 0.2% Tween® 80 in D-PBS and administered by i.t. route. For i.t. administrations animals were anaesthetized with isoflurane (4% in oxygen) and placed in a supine position. With the help of

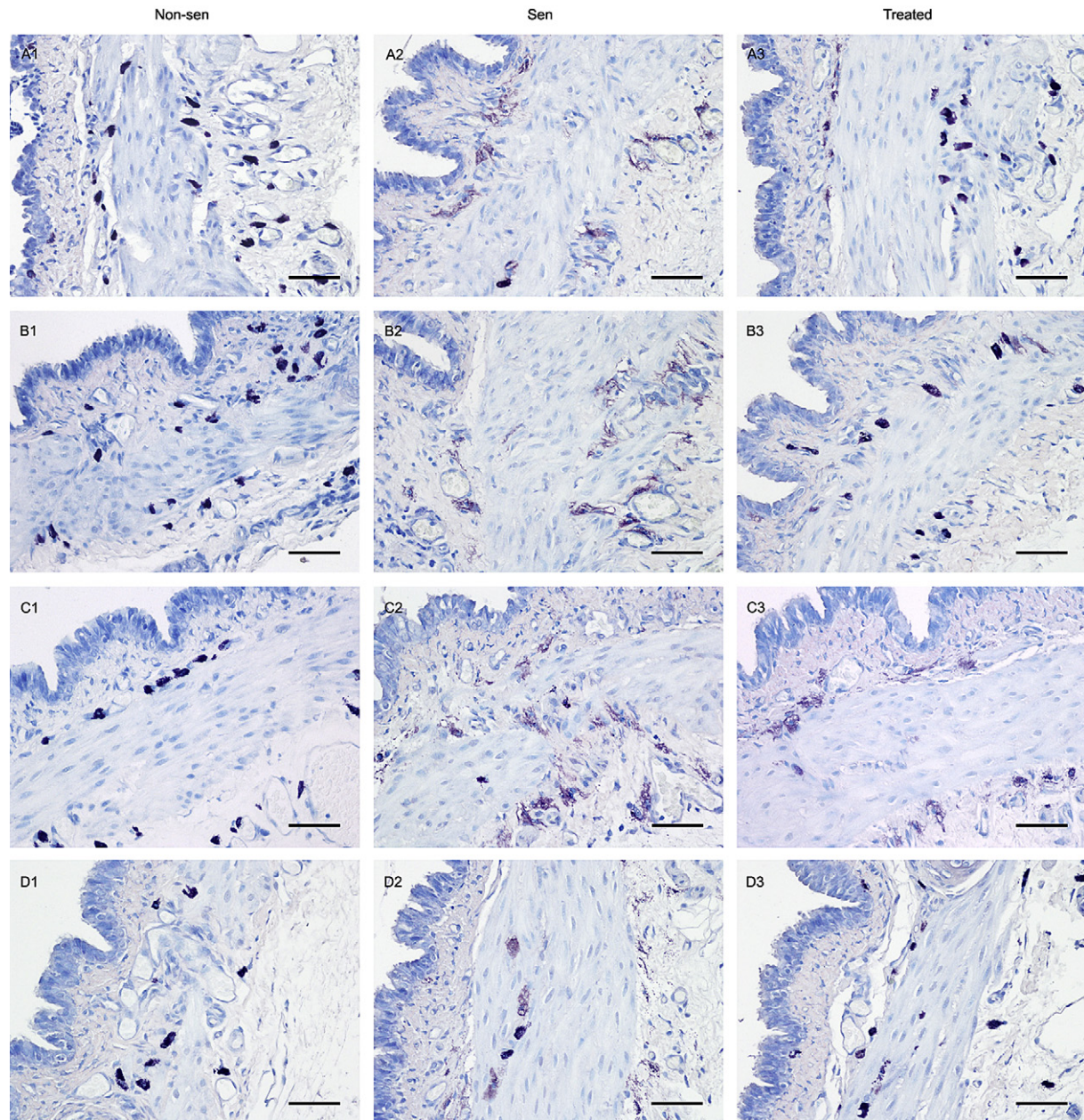


Fig. 3. Pharmacological effect on mast cell degranulation in rat trachea. Animals were passively sensitized with IgE anti-DNP and challenged with DNP 24 h later. Animals were euthanized 5 min post challenge and tracheas were collected for mast cell degranulation quantification. Representative images of toluidine blue-stained tracheal sections obtained from 4 different studies. (A1, B1, C1, D1) Non-treated non-IgE sensitized groups. (A2, B2, C2, D2) Non-treated IgE sensitized groups. (A3, B3, C3, D3) IgE sensitized groups treated with (A3) cromoglycate 10 mg/kg i.v., (B3) cromoglycate 10 mg/kg i.t., (C3) fluticasone 1 mg/kg i.t. and (D3) R406 30 mg/kg p.o.. Cromoglycate was administered 5 min prior to DNP challenge; fluticasone and R406 were administered 1 h before challenge. Scale bars = 50 μ m.

a laryngoscope, the cannula (Microsprayer[®], model IA-1B, rat size, from Penn Century[™]) was introduced through the larynge and positioned just above the *carina* of trachea and 0.2 ml of the solution were delivered into the trachea. According to their PK profile by oral, i.v. or i.t. route all compounds were administered 1 h before DNP challenge except for cromoglycate, which was administered 5 min before challenge due to its extremely short half life [13].

2.6. Data and statistical analysis

Values of extravasated Evans blue and tracheal mast cell degranulation were expressed as mean $\pm$ SEM of 6 animals. For comparison among groups, one-way ANOVA followed by Dunnett's

test was used (GraphPad Prism 5.00, GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when $P < 0.05$.

3. Results

3.1. DNP-induced tracheal plasma extravasation and mast cell degranulation

Challenge with DNP in IgE anti-DNP sensitized rats produced an average of 7-fold increase, ranging between 4 and 11-fold increase, in tracheal Evans blue extravasation as compared to non-sensitized rats (Fig. 1A). The DNP challenge also induced a significant increase

in tracheal mast cell degranulation in sensitized animals (78%) measured histologically, compared with non-sensitized animals, where the majority of mast cells were intact (7% degranulated) (Fig. 1B and C), suggesting that plasma extravasation in the trachea is associated with local mast cell degranulation.

3.2. Pharmacological characterization

In order to pharmacologically characterize the model, the effects on plasma extravasation of a mast cell stabilizer (cromoglycate i.v. and i.t.), an antihistamine (cetirizine p.o.), a cysLT1 antagonist (montelukast p.o.), a corticosteroid (fluticasone i.t.) and a Syk inhibitor (R406 p.o.) were assessed (Fig. 2). The effects on mast cell degranulation in trachea were assessed for those compounds targeting mast cells, cromoglycate and R406, as well as for fluticasone at the dose with maximal effect on plasma extravasation (Figs. 3 and 4). A summary of the results obtained on plasma extravasation and mast cell degranulation for all compounds studied is shown in Table 1.

Cromoglycate i.v. significantly inhibited DNP-induced plasma extravasation in the trachea of sensitized rats with 100% inhibition at 10 mg/kg (Fig. 2A). In line with this result, cromoglycate 10 mg/kg i.v. also inhibited mast cell degranulation after antigen challenge (77% inhibition) (Figs. 3A and 4A). Interestingly, by i.t. route, cromoglycate 10 mg/kg also inhibited significantly tracheal plasma extravasation (Fig. 2B) and mast cell degranulation after antigen challenge (Figs. 3B and 4B) with inhibitions of 74% and 89%, respectively. Fluticasone was administered in a dose range between 0.3 and 3 mg/kg by i.t. route. A slight reduction of plasma extravasation in the trachea was observed at the 3 tested doses without showing any dose response (Fig. 2C). Toluidine blue staining of trachea sections showed that fluticasone had no effect on mast cell

degranulation (Figs. 3C and 4C). Neither cetirizine up to 30 mg/kg nor montelukast up to 100 mg/kg inhibited plasma extravasation in IgE-sensitized rats (Fig. 2D and E), consistent with the fact that the main airway rat mast cell mediator is serotonin [14]. The Syk kinase inhibitor R406 inhibited plasma extravasation dose-dependently showing a maximal inhibition of 69% at 30 mg/kg (Fig. 2F) and significantly inhibited mast cell degranulation (81% inhibition at 30 mg/kg) (Figs. 3D and 4D).

4. Discussion

IgE-dependent mast cell degranulation is the central mechanism in immediate allergic reaction. This response is characterized by marked plasma extravasation into perivascular tissue. The acute response is initiated by the release of stored mast cell mediators, histamine and leukotrienes. The model studied herein shows an increase in tracheal plasma extravasation and mast cell degranulation in IgE anti-DNP sensitized animals after DNP challenge, compared with non-sensitized animals, where mainly intact mast cells are observed in the trachea and no increase in plasma extravasation occurs.

We have characterized both responses by using compounds with different mechanisms of action. The compounds targeting mast cell activation, cromoglycate and R406, inhibited both tracheal mast cell degranulation and plasma extravasation, whereas the compounds that antagonize the effect of mast cell mediators, cetirizine and montelukast, were not able to inhibit plasma extravasation. Fluticasone did not show efficacy either in degranulation or in plasma extravasation.

Our results show that cromoglycate by i.v. route completely inhibited plasma extravasation with concomitant inhibition of mast cell degranulation. *In vivo*, the effect of cromoglycate suppressing

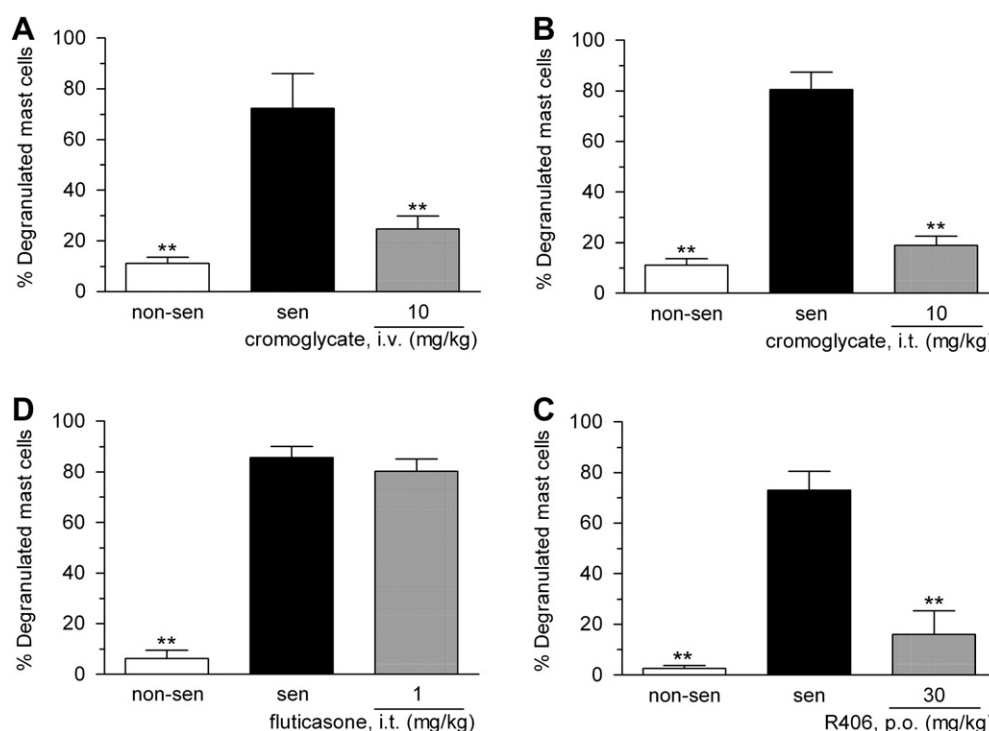


Fig. 4. Effect of (A) cromoglycate i.v., (B) cromoglycate i.t., (C) fluticasone and (D) R406 on mast cell degranulation in the trachea of sensitized rats. Animals were passively sensitized with IgE anti-DNP and challenged with DNP 24 h later. Animals were euthanized 5 min post challenge and tracheas were collected for toluidine blue staining and mast cell quantification. Cromoglycate was administered 5 min prior to DNP challenge by i.v. or i.t. route. Fluticasone was administered by i.t. route 1 h before DNP challenge. R406 was orally administered 1 h before DNP challenge. Non-sen: Non-treated non-IgE sensitized rats; sen: Non-treated IgE sensitized rats. Data are reported as mean $\pm$ S.E.M.; $n = 6$; **, $p < 0.01$ compared with non-treated IgE sensitized group (Dunnett's test).

Table 1

Summary of the effect of tested compounds on plasma extravasation and mast cell degranulation in the trachea of sensitized rats.

	Route of administration	Dose (mg/kg)	Plasma extravasation inhibition (%)	Mast cell degranulation inhibition (%)
Cromoglycate	i.v.	3	51	–
		10	100	77
		30	86	–
	i.t.	3	50	–
		10	74	89
		30	55	–
Cetirizine	p.o.	3	0 (–16)	–
		10	5	–
		30	21	–
Montelukast	p.o.	10	0 (–6)	–
		30	2	–
		100	0 (–14)	–
Fluticasone	i.t.	0.3	18	–
		1	19	7
		3	25	–
R406	p.o.	3	15	–
		10	49	–
		30	69	81

mast cell degranulation has been widely described in the PCA model, where skin mast cells are key players [11,15]. In the present study we also showed that mast cells from trachea were sensitive to cromoglycate. *In vitro*, cromoglycate has been described to modulate differentially the activity and/or function of rat mast cells from different tissues. Cromoglycate dose-dependently inhibited rat peritoneal mast cell degranulation, but it was inactive in rat intestinal mucosal mast cells [16] as well as in the rat basophilic leukemia cell line RBL-2H3 [11,17]. Our results indicate that rat tracheal mast cells respond to cromoglycate in a similar manner as peritoneal mast cells. In comparison to human airway mast cells, our results are in agreement with those previously obtained with mast cells from bronchoalveolar lavage, where cromoglycate showed 50% inhibition at 10 μ M [18]. However, in human disperse lung mast cells cromoglycate was not active up to 1 mM [19]. Taken together, these results emphasize the heterogeneity of mast cells [7,8] and highlight that different pharmacological effects could be obtained depending on the type of mast cells studied.

Interestingly, cromoglycate by i.t. route was also effective at inhibiting tracheal mast cell degranulation and plasma extravasation, being the effects comparable to those obtained by i.v. route. The fact that cromoglycate was effective by both routes, suggests that the DNP-induced tracheal plasma extravasation model is dependent on airway mast cell degranulation and is suitable to test compounds by i.t. route.

The Syk inhibitor R406 was efficacious at inhibiting rat mast cell degranulation and tracheal plasma extravasation. These results are consistent with earlier published data showing that R406 inhibited tryptase and β -hexosaminidase release from cultured human mast cells derived from cord blood and RBL-2H3 cells, respectively [12,20]. The inhibitory effect of R406 in RBL-2H3 cells is comparable to that reported with Syk siRNA in RBL-2H3 cells [21] and highlights the essential role of Syk in mast cell degranulation. The effects observed through inhibition of Syk by R406 points out the potential role of Syk inhibitors as new anti-allergic drugs.

It is well established that there are differences in mast cell mediators depending on the species. In our study, cetirizine and montelukast did not block tracheal plasma extravasation consistent with the fact that serotonin is the major mediator of anaphylactic bronchoconstriction in rats [14]. In line with our results, mepyramine has no effect on antigen-induced airway plasma extravasation and bronchospasm in Brown Norway rats [22]. Regarding

montelukast, it has been previously reported that it significantly reduces ovalbumin-induced microvascular leakage in the trachea of sensitized Brown Norway rats [22]. Discrepancies with our results in Wistar rats are explained by differences between strains.

In our experimental conditions, fluticasone administered 1 h before DNP challenge failed to inhibit plasma extravasation and mast cell degranulation. Different results are obtained when corticosteroids are administered for a long period of time before challenge. Dexamethasone 1 mg/kg i.v. administered 12 h before challenge has been reported to inhibit plasma extravasation and mast cell degranulation in the trachea of OVA-sensitized rats [23]. We also observed partial inhibition of plasma extravasation when fluticasone was administered 24 h prior to antigen challenge (data not shown). These results are consistent with emerging data showing that corticosteroids suppress mast cell degranulation in a time-dependent manner in part through upregulation of inhibitory regulators of Fc ϵ RI signaling [24,25].

5. Conclusions

In summary, the results described herein suggest that airway mast cells play a key relevant role in the DNP-induced tracheal plasma extravasation model. We show that only the mechanisms of action that reduce mast cell degranulation are active at blocking tracheal plasma extravasation, suggesting that this model is dependent on mast cell degranulation. Therefore, the DNP-induced tracheal plasma extravasation model might be useful for the evaluation of new anti-allergic drugs intended to target airway mast cells by different administration routes, including the i.t. one.

Moreover, this model may be convenient in drug discovery due to its robustness, simplicity and suitability for the evaluation of compound duration of action and the establishment of PK/PD relationships.

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