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Pharmacological modulation of allergic inflammation in the rat airways and association with mast cell heterogeneity

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Abstract

Administration of ovalbumin by aerosol to sensitised rats produced a rapid (15 min) protein exudation in different airway tissues, as determined by Evans blue staining. This was associated with marked mast cell degranulation determined by histological examination, with there being no difference between mucosal and connective tissue mast cells. A 5-day administration regimen with compound 48/80 selectively depleted connective tissue mast cell (positive to berberine staining) without modifying ovalbumin-induced plasma protein extravasation. Treatment of rats with dexamethasone (1 mg/kg, −12 h) or nor-dihydroguaiaretic acid (30 mg/kg i.p., −30 min) significantly reduced ovalbumin-induced protein extravasation and preserved mucosal mast cell morphology. Indomethacin (4 mg/kg i.v., −30 min) exerted no effect on either parameter. In conclusion, we propose the mucosal mast cell as a target cell responsible at least partly for the inhibitory actions of known anti-inflammatory drugs. We suggest an involvement of endogenous leukotriene(s), but not prostanoid(s), in mucosal mast cell activation/degranulation. © 2001 Published by Elsevier Science B.V.

Keywords: Dexamethasone; Indomethacin; Nor-dihydroguaiaretic acid; Plasma protein extravasation; Compound 48/80; Mast cell

1. Introduction

The concept of mast cell heterogeneity represents a focal point in recent discussions on mast cell biology. Two distinct mast cell populations exist, with different phenotypes and functions, and likely different functional roles during inflammatory and immune responses (Galli et al., 1999). Connective tissue mast cells contain large amounts of heparin located in intracellular granules, as detected by immunocytochemistry (Oliani et al., 1997) and have been shown to respond promptly to stimulation with compound 48/80 (Stevens and Austen, 1989). This is not seen in rat mucosal mast cells since they are relatively insensitive to stimulation with compound 48/80 and their granules contain a different proteoglycan (chondroitin sulphate). Another difference between the two mast cell types is in the mediators generated in response to activation. For instance, with respect to the catabolism of arachidonic acid, connective tissue mast cells produce mainly prostaglandin D_2, whereas mucosal mast cells are able to generate prostaglandin D_2 as well as leukotrienes B_4 and C_4 (Stevens and Austen, 1989; Galli, 1990).

There is a renewed interest in the role that mast cells play in the initiation and progression of the allergic inflammatory reaction (Galli et al., 1999). Mast cell stabilizers diminish the intensity of the inflammatory response, as seen in several rodent species (Marone et al., 1998). Inhibitors of selective mast cell-derived mediators, such as histamine, reduce the inflammation associated with antigen challenge in sensitized animals (Hessel et al., 1995; Tavares de Lima and Da Silva, 1998). This observation, together with the ability of mast cells to secrete not only vasoactive amines but also several pro-inflammatory cytokines and chemokines, including tumor necrosis factor-α and interleukin-6 (Galli et al., 1999), clearly points to this cell type as an important target for immunomodulation of the host allergic inflammatory reaction.

A few studies have investigated the ability of glucocorticoid hormones and synthetic derivatives to modulate mast cell functions. For instance, glucocorticoids reduce mast cell maturation (Marone et al., 1998) and can also

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affect their response to stem cell factor-1-α and interleukin-3 (Finotto et al., 1997; Marone et al., 1998; Miller et al., 1999). Treatment of rats with dexamethasone inhibits ex-vivo mast cell degranulation in a stimulus-dependent manner (Perretti et al., 1990) and promotes the synthesis of annexin 1 (Oliani et al., 2000). More recently developed anti-allergic drugs also have the potential to alter mast cell functions. This may be particularly true for leukotriene antagonists (Busse et al., 1999) and other inhibitors of eicosanoid synthesis and/or functions (Rao et al., 1993; Marone et al., 1998).

The hypothesis tested in the present study was that anti-inflammatory and anti-allergic drugs may exert their actions at least in part by reducing mast cell activation. We used a model of allergic reaction in the rat and tested the effects of dexamethasone, nor-dihydroguaiaretic acid and indomethacin on (i) the increase in vascular permeability of airway tissues, and (ii) on morphological alterations in the trachea mast cell populations.

2. Material and methods

2.1. Animals

Male Wistar rats (*Rattus norvegicus albinus*, 200–230 g in body weight) came from Biotério Central of Instituto de Ciências Biomédicas of São Paulo University, São Paulo, Brazil. Animals were maintained in a temperature- and humidity-controlled room, with a 12-h light–dark cycle and were allowed food and water ad libitum. The experimental procedures were performed according to the guidelines issued by the University of São Paulo (1998).

2.2. Protocol for animal sensitisation and challenge

Animals were sensitised by injection of 10 μg i.p. ovalbumin (chicken egg albumin crude; grade III, Sigma, St. Louis, MO) suspended in 10 mg aluminium hydroxide. After 14 days, rats ($n = 10$) were challenged with ovalbumin administered by aereosol (1% in sterile saline; 15-min application at 0.75 ml min^{-1}). The saline group of animals ($n = 5$) were sensitised with ovalbumin but challenged with saline. Finally, the basal group of rats ($n = 5$) was left untreated with no sensitisation or challenge.

2.3. Drug treatment

Groups of rats ($n = 5$) were treated with the drugs listed below prior to ovalbumin challenge. The selective connective tissue mast cell degranulator compound 48/80, was given at 10 days before ovalbumin challenge, in increasing doses (initial dose 1 mg/kg and final dose 5 mg/kg i.p.) twice a day for 5 days (Riley and West, 1955). The glucocorticoid dexamethasone was given at a dose of 1 mg/kg i.v. 12 h before challenge (Eum et al., 1996). The 5-lipoxygenase inhibitor, nor-dihydroguaiaretic acid, was used at a dose of 30 mg/kg i.p. (Pretolani et al., 1987), whereas the non-selective cyclo-oxygenase inhibitor, indomethacin, was given at a dose of 4 mg/kg i.v. (Nielsen, 1977). The latter two drugs were given 30 min prior to ovalbumin.

2.4. Measurement of vascular permeability

Determination of vascular permeability was carried out using Evans blue staining (Sigma) as described by Sirois et al. (1988). Briefly, this vital stain was injected i.v. at a dose of 20 mg/kg immediately before ovalbumin-induced challenge. Rats were killed at the end of the 15-min inhalation period and the larynx, trachea, external and internal bronchi removed and wet weighed.

For each tissue, fragments were taken and incubated at 60 °C for 120 h to determine the dry weight. Other tissue portions were fixed in 4 ml of formamide (Sigma) per gram of tissue, and the staining was extracted by 24-h incubation at room temperature. Subsequently, samples were centrifuged, and the absorbance of the supernatants was measured at 620-nm wavelength in a spectrophotometer (Titertek™ Multiskan, Flow Laboratories, Finland). The Evans blue present in the samples was calculated from a standard curve constructed with known concentrations of the stain (0.3–100 μg/ml).

2.5. Histological analysis

Segments of trachea were collected 15 min after ovalbumin challenge and immediately fixed with 2% paraformaldehyde, 2% glutaraldehyde in Sorensen phosphate buffer, pH 7.4 at 4 °C for 24 h (Oliani et al., 1997). Following embedding in historesin (Technovit 7200, TAAB Laboratories, UK), sections (2 μm) were cut and stained with 1% toluidine blue in 1% borax solution (TAAB Laboratories).

To differentiate between mucosal and connective tissue mast cells, some sections from the experimental groups were stained with 0.02% berberine sulfate (Fluke), pH 3.5, for 20 min, mounted in glycerol (Girol et al., 1996) and analysed with an Olympus BH-2-RFCA model fluorescence microscope at a wavelength of 435 nm. Then, the same sections were stained with 1% toluidine blue in 1% borax solution, with the same field being re-photographed.

2.6. Data handling and statistical analysis

Vascular permeability is expressed as μg Evans blue extracted per mg of dry weight tissue. For quantification of the histological preparations, firstly, an observer unaware of the treatment counted the number of mast cells in each trachea section, and the percentage of degranulated mast cells was subsequently quantified. Under a high-power objective (×40), the number of mast cells was counted in

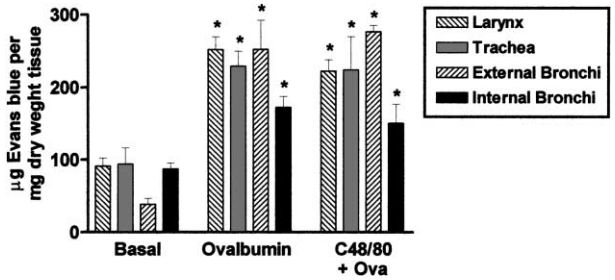

Fig. 1. Evans blue extravasation in sensitised rats following antigen challenge: lack of effect of compound 48/80. Rats were sensitised to ovalbumin (see Section 2) and challenged with saline or ovalbumin for 15 min (delivered by aerosol). Evans blue (20 mg/kg) was injected i.v. immediately before challenge to monitor vascular permeability. Some rats were pretreated with compound 48/80 prior to ovalbumin challenge. In all cases, animals were killed at the end of the challenge period, tissues were collected and Evans blue extracted. Results are means ± S.E.M. of five rats per group. * $P < 0.05$ vs. saline-challenge group.

three areas of approximately 100 μm^2 (Lidegran et al., 1996), using 10 serial sections (2-μm each, leaving 15-μm space between each section) from each trachea ($n = 5$ per group). In all cases, data are means ± S.E.M.

Differences among groups were determined on original values using analysis of variance followed by the Bonferroni test. Differences were considered significant at *P*-values less than 0.05.

3. Results

3.1. Effects of antigen inhalation

Challenge of non-sensitised basal rats produced extravasation of Evans blue (Fig. 1). This vascular permeability was not significantly different from that of the sensitised animals challenged with saline only (data not show). However, a significant increase could be measured in rats challenged with the antigen (Fig. 1). Ovalbumin challenge caused a two-to four-fold increase in the amount of extracted Evans blue measured in different sections of the airways. A small increase, though significant, was measured in the internal bronchus, whereas external bronchi had the highest increase in vascular permeability. A similar pattern of extravasation was measured for the larynx and the trachea (Fig. 1).

3.2. Analysis of mast cell heterogeneity

The total number of mucosal and connective tissue mast cells counted in the trachea sections ranged between 3–6 and 6–10 cells per section, respectively (Table 1).

Light microscopy confirmed the presence of intact metacromatic mast cells in the trachea of basal rats. The distinction between mucosal mast cells and connective tissue mast cells was anatomical, with the former cell type being localized in the mucosal layer near the epithelium, and the latter cell type being found in smooth muscle and adventitious layers (Fig. 2A). The two types were also distinguishable by cell size and intense metachromatic granules which, after the berberine reaction, were fluorescent in connective tissue mast cells (Fig. 3A and B). A high incidence of degranulated mucosal and connective tissue mast cells was observed in sections prepared from ovalbumin-challenged rats (Fig. 2B). Mast cell degranulation was identified by the clear presence of metachromatic granules in the extracellular matrix around the mast cell (compare Fig. 2A with Fig. 2B).

Histological examination of mast cell heterogeneity in the trachea from the different groups of animals was carried out and statistical evaluation was made (Table 1). Whereas mast cells were virtually intact in basal rats, a marked incidence of degranulation was seen in the group of rats subject to ovalbumin challenge. Moreover, the

Table 1
Cumulative effect of pharmacological modulation of mast cell morphology in the rat trachea following antigen challenge

Rat	Mucosal mast cell (cell per 100 μm^2)			Connective tissue mast cell (cell per 100 μm^2)		
	Intact	Degranulated	%Dgr	Intact	Degranulated	%Dgr
Basal	4.8 ± 0.9	0	0	7.1 ± 0.6	0.3 ± 0.2	4
OVA	1.5 ± 0.3	3.3 ± 0.8	69	2.3 ± 0.6	5.8 ± 0.9	71
C48/80 + OVA	1.8 ± 0.2[a]	1.9 ± 0.7[a]	49	0	0	–
DEXA + OVA	4.0 ± 0.4	0[a]	0	7.8 ± 1.0	0.3 ± 0.6[a]	3
NDGA + OVA	3.2 ± 0.6	0.2 ± 0.2[a]	6	7.6 ± 0.7	0.4 ± 0.2[a]	5
INDO + OVA	1.6 ± 0.3[a]	1.6 ± 0.3[a]	50	2.4 ± 0.7	4.6 ± 0.4	65

Data refer to the no. of each mast cell type per 100 μm^2 area (2-μm sections) and are mean ± S.E.M. from three areas per section (total of 10 serial sections per animal) of trachea stained. The presence of intact and degranulated (Dgr) mast cells and the relative percentages are shown. All groups were formed by five rats, with the exception of the ovalbumin control group (OVA) ($n = 10$). Drug pre-treatment, subsequent to ovalbumin challenge (+OVA), was as follows: compound 48/80 (C48/80) 2 mg/kg per day for 5 consecutive days; dexamethasone (DEXA) 1 mg/kg s.c., −12 h; nor-dihydroguaiaretic acid (NDGA) 30 mg/kg i.p., −30 min; indomethacin (INDO) 4 mg/kg i.v., −30 min.

[a] $P < 0.05$ vs. ovalbumin-challenge control group.

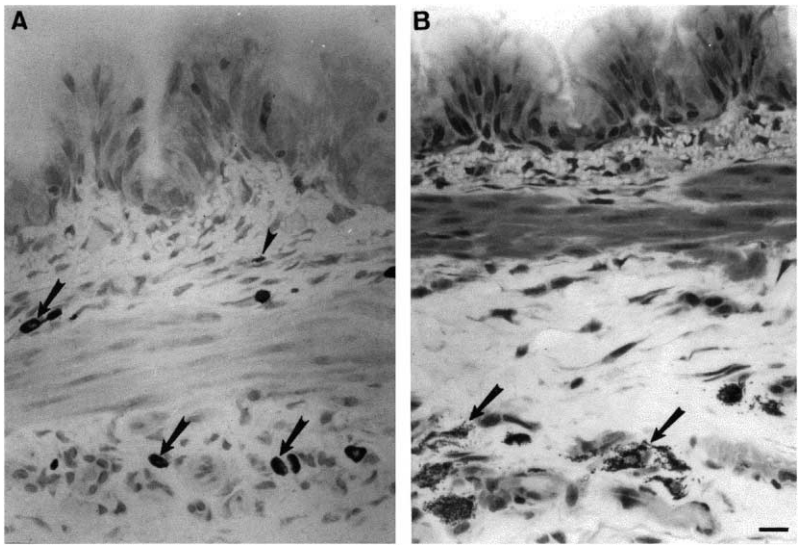

Fig. 2. Determination of mast cell integrity in rat trachea following ovalbumin challenge. (A) Tracheal sections from rats from the basal group. Mast cells with metachromatic granules as seen at the mucosal (arrowhead) and adventitious (arrows) layers. (B) Tracheal sections from the ovalbumin-challenged rats showing degranulated mast cells (arrows). Sections (2 μm) were stained with toluidine blue. Bar = 20 μm.

degree of degranulation in mucosal and connective tissue mast cells was similar, with values of 69% and 71%, respectively.

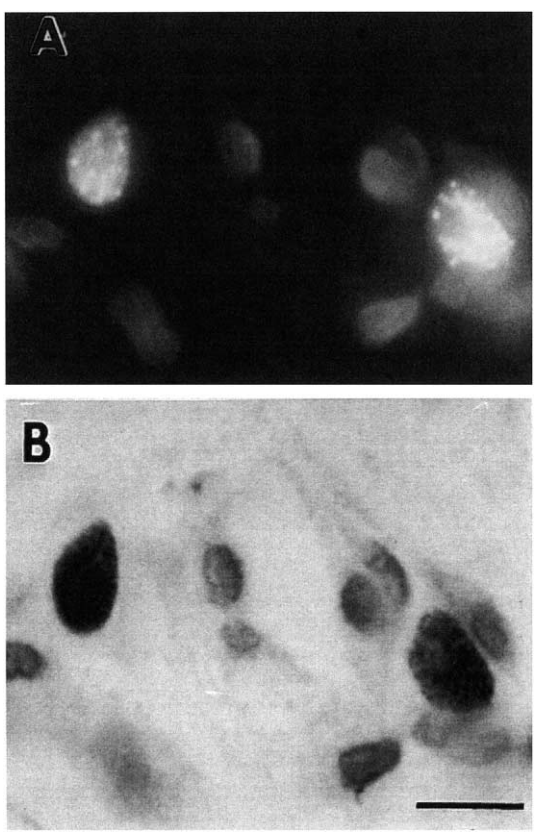

Fig. 3. Identification of connective tissue mast cells following staining with berberine sulphate. (A) Tracheal connective tissue mast cells were stained with berberine sulphate and showed intense fluorescence associated with cytoplasmic granules. (B) The same section was stained with toluidine blue, confirming the identity of the metachromatic cytoplasmic granules. Bar = 10 μm.

After a 5-day treatment, compound 48/80 did not modify the increase in plasma protein extravasation produced by antigen challenge in any of the rat tissues analysed (Fig. 1). Similarly, no significant difference in the extent of degranulation of mucosal mast cells could be measured (Table 1). As expected, this regimen of administration with compound 48/80 abolished the detection of connective tissue mast cells (Table 1).

3.3. Pharmacological modulation of ovalbumin-induced allergic reaction

Treatment of rats with dexamethasone significantly reduced the increase in vascular permeability produced by

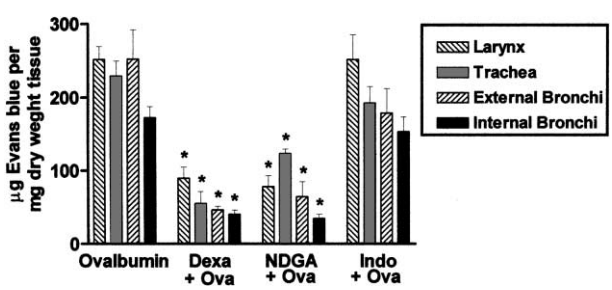

Fig. 4. Pharmacological modulation of Evans blue extravasation in sensitised rats following antigen challenge. Rats were sensitised to ovalbumin (see Section 2) and challenged with ovalbumin for 15 min (delivered by aerosol). Evans blue (20 mg/kg) was injected i.v. just before challenge to monitor vascular permeability. Animals were left untreated (ovalbumin group, $n = 10$) or treated with dexamethasone (Dexa; 1 mg/kg i.v., −12 h, $n = 5$), with nor-dihydroguaiaretic acid (NDGA; 30 mg/kg i.p., −30 min, $n = 5$) or with indomethacin (Indo; 4 mg/kg i.v., −30 min, $n = 5$) prior to antigen challenge. In all cases, animals were killed at the end of challenge period, tissues were collected and Evans blue was extracted. Results are means ± S.E.M. of n rats per group. $^{*}P < 0.05$ vs. ovalbumin-challenge group.

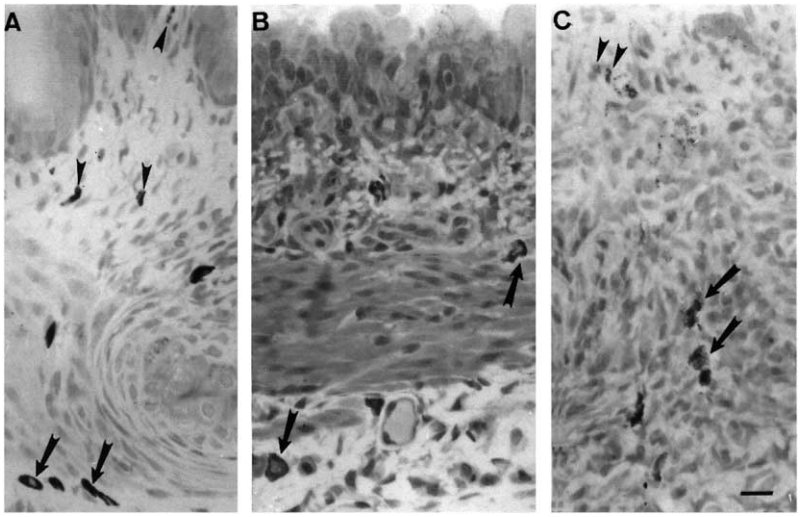

Fig. 5. Pharmacological modulation of mast cell integrity in rat trachea following ovalbumin challenge. (A) Tracheal sections of the dexamethasone group illustrating the presence of intact mucosal and connective tissue mast cells (arrowheads and arrows, respectively). (B) As in A, but after nor-dihydroguaiaretic acid pretreatment (arrows indicate representative intact mast cells). (C) Presence of degranulated mucosal and connective tissue mast cells after pretreatment with indomethacin (arrowheads and arrows, respectively). Drugs were given as in Fig. 3. Sections (2 μm) were stained with toluidine blue. Bar = 20 μm.

antigen challenge in all tissues under observation (Fig. 4). In some cases, Evans blue extravasation was lower that measured in tissues from basal rats (compare Figs. 1 and 4 for trachea and internal bronchi). The 5-lipoxygenase inhibitor nor-dihydroguaiaretic acid produced similar results with the following inhibition of Evans blue extravasation: 71 ± 6% for the larynx; 64 ± 3% for the trachea; 82 ± 8% for external bronchi; and 82 ± 2% for internal bronchi (Fig. 4).

The inhibitory effects produced by these two drugs are reflected by the stabilization of tissue mast cells. Fig. 5 shows representative sections in which the integrity of mucosal and connective tissue mast cells can be seen after either treatment (Fig. 5A and B). The cumulative data of the histological analysis are given in Table 1. Dexamethasone and nor-dihydroguaiaretic acid had a pronounced inhibitory effect on mucosal and connective tissue mast cell degranulation. Nor-dihydroguaiaretic acid appeared to be even more effective than the glucocorticoid, at least in preventing mucosal mast cell activation.

In contrast to dexamethasone and nor-dihydroguaiaretic acid, treatment of rats with indomethacin did not significantly affect ovalbumin-induced plasma protein extravasation and mast cell activation in the rat trachea (Fig. 5 and Table 1).

4. Discussion

In the present study, we examined the rapid exudative response produced by antigen challenge in airway tissue a response associated with mast cell degranulation. Of the two mast cell types, mucosal mast cells appear to be functionally more important for the production of this rapid inflammatory response than connective tissue mast cells. In fact, the data obtained with compound 48/80 exclude a relevant functional involvement of connective tissue mast cells in the ovalbumin-induced increase in vascular permeability. Pharmacological treatment with anti-inflammatory drugs prevented both plasma protein extravasation and mast cell degranulation. We suggest that the stabilisation of mucosal mast cell may be responsible, at least in part, for the protective action afforded by dexamethasone and nor-dihydriguaiaretic acid in this model and, possibly, in other forms of allergic reaction.

Mast cells are strategically distributed in several tissues and microvascular beds. This cell type is often seen in close proximity to capillaries and post-capillary venules (Kubes and Granger, 1996). Mast cell activation followed by the release of mediators is responsible for the rapid vascular responses observed in the initial stages of the inflammatory reaction (Yang et al., 1999). Since more recent studies have highlighted the ability of these cells to synthesize de novo several pro-inflammatory cytokines (Galli et al., 1999), it is now clear that mast cells may also have a prominent role in the pathogenesis of persistent host inflammatory/allergic reaction. This is also substantiated by experiments with mast cell-deficient Ws/Ws rats (Nishida et al., 1998).

The availability of compound 48/80, a selective degranulator of connective tissue mast cells (Galli, 1990; Tavares de Lima and Da Silva, 1998), has allowed at least a partial characterisation of the physio-pathological role that each mast cell type plays in models of experimental pathology. Compound 48/80 targets connective tissue mast cells by increasing intracellular calcium concentrations,

hence inducing the release of histamine and the generation of reactive oxygen species (Fukuishi et al., 1997). However, the mechanistic explanation for the selectivity of compound 48/80 for connective tissue mast cells remains elusive, though the presence of a specific receptor may be plausible. In the present study, we administered compound 48/80 according to a well-validated dosing regimen (Tam et al., 1988) to produce a virtual disappearance of connective tissue mast cells in airway tissues. The effectiveness of the treatment with this compound was confirmed by histological examination of the trachea, where connective tissue mast cells could not be detected. Nonetheless, the extravasation of Evans blue produced by ovalbumin challenge was unaffected.

Furthermore, data regarding the specificity of the actions of compound 48/80 are available. Following a 5-day treatment protocol, as used in the present study, compound 48/80 depletes the stocks of histamine and serotonin in connective tissue mast cells (Di Rosa et al., 1970), and prevents the first hour of carrageenin-induced rat paw edema (Di Rosa et al., 1970). In contrast, the action of compound 48/80 does not lead to degranulation of mucosal mast cells (Enerbäck, 1966; Lidegran et al., 1996). Finally, Kubes and Granger (1996) have demonstrated that compound 48/80 is unable to directly activate either neutrophils or endothelial cells.

Finally, a recent study by Ikawati et al. (2000) showed that compound 48/80 contracts isolated tracheal segments prepared from control rats (+/+) but not from mast cell-deficient Ws/Ws animals. Our data show that mucosal mast cells exert a major role in the increase in vascular permeability. In fact, mucosal mast cell release several mediators, which are potential candidates to regulate the allergic reaction, including lipid mediators (leukotriene B_4, leukotriene C_4, prostaglandin D_2), histamine and serotonin (Heavey et al., 1988; Miller et al., 1999).

The causal relationship between mucosal mast cells and the initial phase of the allergic reaction is supported by experiments with known pharmacological tools. Glucocorticoid hormones are potent anti-inflammatory and anti-allergic drugs and are effective in virtually all forms of experimental allergic reaction. In addition, corticosteroids are used as life-saving drugs in children and adults with asthma (Nikolaizik et al., 1997). When the potent synthetic glucocorticoid dexamethasone was administered to rats, a significant attenuation of Evans blue extravasation into the larynx, trachea, external and internal bronchi was observed. This was associated with a marked inhibition of mast cell degranulation. Whereas the potent effect of steroids on the humoral and cellular response of the host inflammatory reaction has already been reported in several other experimental systems (especially at the dose used of 1 mg/kg) (Sewell et al., 1998; Pasquale et al., 1999), the effect on mast cell degranulation warrants further investigation.

Glucocorticoid hormones have been reported to affect mast cell maturation and re-invasion into tissues (e.g. skin or peritoneal cavity, see Soda et al., 1991; Finotto et al., 1997). This is likely to be linked to inhibition of the synthesis of stem cell factor-1 and interleukin-3 (Finotto et al., 1997; Marone et al., 1998; Miller et al., 1999). There have been conflicting reports concerning the capacity of dexamethasone to inhibit mast cell degranulation, but this is particularly true in vitro. In a previous ex vivo study, we observed a stimulus-dependent inhibitory effect of dexamethasone. The steroid was given in vivo, and it was unable to affect compound 48/80-induced ex vivo degranulation of rat connective tissue mast cells, but did inhibit the response produced by phospholipase A_2 (Perretti et al., 1990). Using histological examination of lung tissues, we demonstrated here that the integrity of mucosal and connective tissue mast cells is maintained following in vivo treatment with a single dose of dexamethasone. It is tempting to propose that this effect of dexamethasone on mast cells is crucial for its capacity to affect the initial phase of the experimental allergic reaction in rat airway tissues.

The inhibitory effect of nor-dihydroguaiaretic acid, consistent with the lack of effect of indomethacin, on the increased vascular permeability points to the role that leukotrienes have in mediating mast cell degranulation in this experimental model of inflammation. Nor-dihydriguaiaretic acid was almost equipotent to dexamethasone regarding all parameters under observation. The only innovative therapeutic intervention for asthma in recent years has been the development of specific leukotriene B_4 antagonists (Marone et al., 1998). We would therefore like to propose that mast cells may have a contributory role to the production of these mediators and, conversely, drugs able to block mast cell degranulation may be of therapeutic use. There is experimental evidence that leukotrienes produced by mast cells play a role in eosinophil recruitment (Harris et al., 1997). Leukotriene synthesis occurs in lipid-rich cytoplasmic organelles, known as lipid bodies, which are reservoirs of phospholipids and consequently serve as non-membrane sources of the substrate, arachidonic acid (Dvorak, 1997). Nor-dihydriguaiaretic acid was initially developed as a selective 5-lipoxygenase inhibitor (Boctor and Pugsley, 1986); however, its selectivity was subsequently questioned, and inhibitory actions on the formation and release of prostanoid have been reported (Igarashi et al., 1993; Rodgers and Xiong, 1997). In the present study, the lack of effect of indomethacin on plasma extravasation can be explained by an increase in substrate availability for the 5-lipoxygenase pathway. This possibility for the metabolic usage of arachidonic acid has also been suggested by others, including ourselves (Adcock and Garland, 1980; Tavares de Lima and Da Silva, 1998). On the basis of these observations, we clearly suggest that nor-dihydriguaiaretic acid is active via blockade of leukotriene synthesis. This is also in line with the effect produced by the synthetic glucocorticoid.

Finally, the hypothesis put forward by the present study is reinforced by the observation that mucosal mast cells can synthesise leukotrienes, whereas connective tissue mast cells mainly produce prostanoids (Heavey et al., 1988; Murakami et al., 1997; Marshall et al., 1999). We suggest that leukotrienes B_4 and C_4 mediate the rapid increase in vascular permeability measured after antigen challenge. Mucosal mast cells are targeted by steroids and leukotriene synthesis inhibitors to inhibit the rapid exudative response measured in sensitised rats following antigen challenge.

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