

BACKGROUND: Rat trachea display a differential topographical distribution of connective tissue mast cells (CTMC) and mucosal mast cells (MMC) that may imply regional differences in the release of allergic mediators such as tumor necrosis factor- α (TNF- α) and eicosanoids.

Aim: To evaluate the role of CTMC and MMC for release of TNF- α and eicosanoids after allergenic challenge in distinct segments of rat trachea.

Materials and methods: Proximal trachea (PT) and distal trachea (DT) from ovalbumin (OVA)-sensitized rats, treated or not with compound 48/80 (48/80) or dexamethasone, were incubated in culture medium. After OVA challenge, aliquots were collected to study release of TNF- α and eicosanoids.

Results: Release of TNF- α by PT upon OVA challenge peaked at 90 min and decayed at 6 and 24 h. Release from DT peaked at 30–90 min and decayed 6 and 24 h later. When CTMC were depleted with 48/80, OVA challenge exacerbated the TNF- α release by PT at all time intervals, while DT exacerbated TNF- α levels 6 and 24 h later only. Dexamethasone reduced TNF- α production after 90 min of OVA challenge in PT and at 3 and 6 h in DT. OVA challenge increased prostaglandin D₂ in DT and leukotriene B₄ in both segments but did not modify prostaglandin E₂ and leukotriene C₄ release.

Conclusion: OVA challenge induces TNF- α release from MMC, which is negatively regulated by CTMC. The profile of TNF- α and eicosanoids depends on the time after OVA challenge and of the tracheal segment considered.

Key words: TNF, Eicosanoids, Mast cells, Airways, Asthma, Rat

Differential regulation of the release of tumor necrosis factor- α and of eicosanoids by mast cells in rat airways after antigen challenge

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Introduction

Asthma is an inflammatory airways disease characterized by migration of inflammatory cells to the airways, bronchoconstriction and bronchial hyper-responsiveness (BHR).¹ During its initial phases, activated mast cells release a wide spectrum of preformed and newly generated mediators including histamine and 5-hydroxytryptamine, tumor necrosis factor- α (TNF- α), arachidonic acid (AA) metabolites, proteases and nitric oxide.² The release of those mediators induces different pathophysiologic effects, including bronchoconstriction and excessive mucus production and secretion associated with respiratory disorders.³ In addition, these mediators have an important role in regulating the subsequent chain of pulmonary inflammatory events.⁴

One of the major mast cell-derived cytokines is TNF- α and its effects on airway inflammation are well recognized.⁵ Generation of TNF- α was also demonstrated in the bronchoalveolar lavage fluid from

antigen-challenged animals and from asthmatic patients.^{6–8} TNF- α receptor antagonists have been shown to prevent BHR in a model of allergic inflammation.⁹ TNF receptors have been identified in human airway smooth muscle¹⁰ and, accordingly, TNF- α might play a role in regulating the intrinsic properties of airway smooth muscles. This may account for the fact that isolated trachea incubated with TNF- α are more responsive to cholinergic stimuli.^{11,12} Preformed immunoreactive TNF- α has been observed within the granules of mast cells, indicating the likelihood of its rapid release after mast cell activation.^{13–16} AA metabolites such as prostaglandins (PGs) and leukotrienes (LTs) are also vigorously released after mast cell activation.¹⁷ PGs display a number of stimulatory and/or inhibitory effects on airway smooth muscle. For instance, PGD₂ induces eosinophil recruitment, bronchoconstriction and BHR.¹⁸ On the contrary, PGE₂, which is also released by airway epithelial and smooth muscle cells, acts as a protective relaxing prostanoid,^{19,20} as

does PGE₁ under conditions where PGI₂ is inactive and only suppresses aggregation of circulating platelets.²¹ Furthermore, PGE₂ exerts potent effect on airway epithelial wound closure,²² suggesting a protective role on epithelial damage such as observed in asthma.

Leukotrienes are also important participants in the allergic response of the airways.²³ Once released, they induce mucus secretion, and increase vascular permeability, leukocyte recruitment and potent airway smooth muscle contraction.²⁴ Antigen challenge induces airway epithelial and smooth muscle cell hypertrophy, which is presumably mediated by LTC₄ and LTD₄.^{25–27}

Two distinct mast cell phenotypes are well characterized: connective tissue mast cells (CTMC) and mucosal mast cells (MMC).²⁸ These variants differ in many aspects, including in their responses to drugs and secretagogues.^{2,29,30} Thus, CTMC are sensitive to depletion by compound 48/80,³¹ whereas dexamethasone prevents MMC activation. Cytoplasmic granules of CTMC are rich in heparin and histamine, whereas in MMC the cytoplasmic granules contain the proteoglycan chondroitin-6-sulphate but low concentrations of histamine.² Another difference between MMC and CTMC is the pattern of AA-derived metabolites. The predominant AA metabolite of CTMC is PD₂, whereas MMC generate predominantly lipoxygenase products such as LTD₄ and LTE₄.^{32,33}

A differential distribution of CTMC and MMC on rat trachea has been described.³⁴ Isolated distal tracheal segments are hyper-responsiveness to antigen challenge as compared with proximal tracheal segments,³⁵ a fact that was attributed to differences in mast cell distribution.³⁴ Therefore, it is plausible that anatomical organization of airways leads to physiologically relevant regional differences related to the heterogeneity of mast cells. Since airways are rich in CTMC and MMC, we decided to investigate the potential influence of the mast cell heterogeneity in rat trachea on the release of TNF- α and of eicosanoids, after *in vitro* antigen challenge of proximal and distal tracheal segments.

Materials and methods

Animals

Male Wistar rats (180–220 g) from our departmental animal facilities were housed in plastic cages (18.5 cm \times 18.5 cm \times 13.5 cm), five rats per cage, in a light/temperature-controlled room (12 h/12 h, 21 \pm 2°C). All experiments received prior approval from the Animal Care Committee from the Institute of Biomedical Sciences of the University of São Paulo.

Sensitization

The rats were sensitized by an intraperitoneal injection of 10 μ g of ovalbumin (OVA) mixed with 10 mg of aluminum hydroxide (day 0). One week later (day 7), the rats were boosted with 10 μ g of OVA dissolved in saline by a subcutaneous injection. The OVA challenge was carried out in isolated segments from proximal and distal trachea 14 days later.

Isolation and incubation of tracheal segments

After 14 days of sensitization, the rats were killed under deep chloral hydrate anesthesia (> 400 mg/kg, intraperitoneally), exsanguinated by cutting the neck vessels and the thoracic cavity, then opened. The trachea was removed and dissected free of connective tissue before being divided into two portions of similar weight (21.3 \pm 2.0 mg). Tracheal rings were designated as proximal (corresponding to the first three to five cartilaginous rings closest to the larynx) or distal (corresponding to the last three to five cartilaginous rings closest to the carine) segments.³⁵ The segments of trachea were put into 24-well plastic microplates containing 1 ml of RPMI 1640 medium enriched with 10% fetal calf serum and maintained under 5% CO₂ atmosphere at 37°C.

Antigen challenge

After 60 min of equilibrium, the antigen challenge was performed by OVA addition (final concentration, 100 μ g/ml) into the tracheal cultures. Aliquots of the supernatant (100 μ l) were collected after 30 and 90 min as well as after 3, 6 and 24 h, and stored at –20°C until further use.

Determination of TNF activity

The levels of TNF were measured in aliquots from supernatant of tracheal culture using the L-929 cytotoxicity assay.³⁶ In brief, properly diluted samples (100 μ l) were added to the 96-well microplates containing target L-929 cells (5 \times 10⁴ cells/100 μ l) in the presence of actinomycin D (final concentration, 5 μ g/ml) and incubated during 20 h at 37°C. The supernatants were discarded and the remaining viable adherent cells were stained with crystal violet (0.05%) during 15 min. The absorbance was read at 620 nm (ELISA Titertek Multiskan, Lab Systems, Helsinki, Finland). The TNF titer (U/ml) was defined as the reciprocal of the dilution, which induced 50% of L-929 cell lysis.

Eicosanoid release

In this series of experiments we quantified the release of eicosanoids in selected times after OVA challenge.

To this purpose, using an enzyme immunoassay,³⁷ the concentrations of PGE₂, PGD₂, LTB₄ and LTC₄ were determined 30 and 90 min after OVA challenge. In brief, during 18 h, properly diluted samples of supernatant (100 µl) from tracheal cultures were incubated with acetylcholinesterase-conjugated eicosanoid plus the specific antiserum, in 96-well microtiter plates coated with anti-immunoglobulin G immunoglobulin. Two hours after acetylcholine addition, the optical density was determined at 412 nm in a microplate reader and the concentration of eicosanoids calculated using standard curves.

Effects of pharmacological treatments on OVA-induced TNF production

Compound 48/80. After 10 days of OVA sensitization, groups of rats received 100 µg of compound 48/80 in the morning and evening of the first day, increasing in 100 µg increments to 500 µg in the morning and evening of the fifth day. According to the literature, such a protocol of administration of compound 48/80 depletes up to 90% of CTMC rat trachea.³⁴

Dexamethasone. Groups of sensitized rats were treated by intraperitoneal injection of dexamethasone (1 mg/kg) 18 h before killing the animals. The OVA challenge and the quantification of the levels of TNF were performed as already indicated.

Fixation processing and embedding for light microscopy

After 24 h of *in vitro* treatment, fragments of trachea were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, 0.1 M of sodium cacodylate buffer (pH 7.4) for 2 h at 4°C, washed in sodium cacodylate, dehydrated through a grade series of ethanol, and embedded in UNICRYLth resin (British BioCell International, Cardiff, UK) for light microscopy analysis and for immunohistochemistry. Sections (1.5 µm thick) were cut on an ultramicrotome (Reichert ULTRACUT UCT-GA, Leica, Wien, Austria) and stained with toluidine blue 1% in 0.1 M of sodium cacodylate buffer.

Immunohistochemistry

To analyze the proteoglycan heparin in the CTMC and MMC, and confirm the presence of this in the former mast cell type, UNICRYL sections (1 µm thick) were prepared in histological slides and incubated with the followed reagents: washed in 0.1 M of Tris buffer (Sigma Chemical Co., Poole, UK) (pH 8.2) for 30 min with 3% hydrogen peroxide, then washed again three times in Tris buffer for a further 30 min.

Non-specific binding sites were blocked with 10% chicken egg albumin in Tris buffer (TBAE). The degree of mast cell differentiation was analyzed with the serum monoclonal antibody anti-heparin (ST-1).³⁸ The sections were incubated overnight at 4°C. As a control of the reaction, sections were incubated with non-immune IgM mouse serum (Sigma Chemical Co.) instead of the primary antibody. Sections were washed in 1% TBAE for 40 min before incubation for 1 h at room temperature with the secondary antibody, an anti-mouse IgM (Fc fragment specific) antibody conjugated to 5 nm colloidal gold (1:100; British BioCell International, Cardiff, UK). Sections were washed in Tris buffer (30 min) and washed in distilled water (30 min). Silver enhancing solution SEKL (British BioCell International) was used to augment gold particle staining.³⁹ At the end of the reaction, sections were washed thoroughly in distilled water, counterstained with hematoxylin and eosin and mounted in BIOMOUNT (British BioCell International). Finally, serial sections of trachea were stained with 1% toluidine blue in 1% sodium borate solution, and the same field being re-photographed on the ZEISS-AXIOSKOP2 (Zeiss, Mot, Göttingen, Germany) microscope.

Data handling and mast cell quantification

For quantification of the histological preparations, first an observer unaware of the treatment counted the number of mast cells in each tracheal section, and the percentage of degranulated mast cells was subsequently quantified. Data were determinate using a high-power objective (x 40), and the number of mast cells was counted in three areas of approximately 100 µm² using three serial sections (1.5 µm each, leaving 20 µm space between each section) from each tracheal segment (*n* = 5 per group). In all cases, data are expressed as mean ± standard error of the mean.

Results

Analysis of tracheal mast cell heterogeneity and of the effects of OVA challenge

The presence of metachromatic granules in rat tracheal mast cells was determined by light microscopy after toluidine blue staining. Initially, the distinction between CTMC and MMC was based on their anatomical distribution. The CTMC were found in smooth muscle and adventitia (Fig. 1A), whereas MMC were localized mainly in the mucosal layer, near the epithelium (Fig. 1A). CTMC could be also identified by their size and intense metachromatic cytoplasmatic granules that were highlighted for immunostaining with the anti-heparin monoclonal

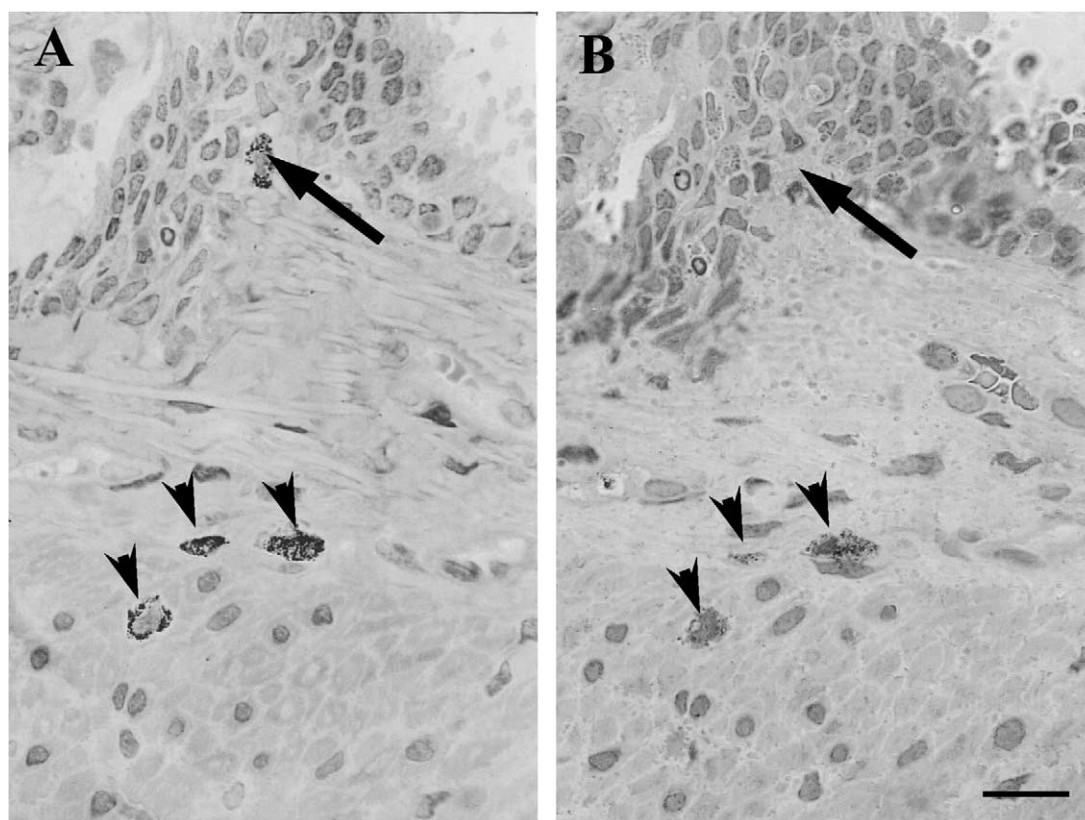


FIG. 1. Morphology of MMC and CTMC in rat trachea tissue sections. (A) Tracheal MMC in the basal group showed metachromatic cytoplasmic granules in the mucosal layer (arrow), whereas the CTMC subtype was predominantly localized in smooth muscle and adventitious layers (arrowheads). Toluidine blue stain. (B) Presence of ST-1 (anti-heparin monoclonal antibody) immunoreactivity in CTMC (arrowheads) and absence of staining in MMC (arrow). Hematoxylin and eosin stain. Bar, 10 μ m.

antibody ST-1 (Fig. 1B). MMC were not immunoreactive to this antibody (Fig. 1B).

After OVA challenge, a high number of degranulated CTMC (proximal trachea, 90%; distal trachea, 91%) and MMC (proximal trachea, 91%; distal trachea, 77%) (Tables 1 and 2) were observed in sections. Mast cell degranulation was identified by the presence of metachromatic granules in the extracellular matrix around the cell.

Proximal and distal tracheal segments differentially release TNF- α after OVA challenge

Fig. 2 shows the time-dependent TNF- α activity found in supernatants from segments of proximal and distal trachea collected from sensitized rats and challenged *in vitro* with OVA. Both proximal and distal segments (hatched and solid bars, respectively) provided a bell-shaped curve for TNF- α activity. Thirty minutes after challenge, the levels of TNF- α

Table 1. Cumulative data for mast cell numbers in rat proximal trachea

	CTMC			MMC		
	Intact	Degranulated	% Degranulated	Intact	Degranulated	% Degranulated
Saline	4.25 \pm 0.06	0	0	6.7 \pm 0.8	0	0
C48/48	n.d.	n.d.	n.d.	6.9 \pm 0.2	0	0
OVA	0.4 \pm 0.2*	3.4 \pm 0.5*	90	0.6 \pm 0.4*	5.8 \pm 0.6*	91
C48/48+OVA	n.d.	n.d.	n.d.	0.9 \pm 0.2**	5.4 \pm 0.5**	86
DEXA+OVA	2.8 \pm 0.2**	1.7 \pm 0.5**	38	3.9 \pm 0.5**	3.1 \pm 0.2**	44

Extent of intact and degranulated CTMC and MMC (as measured in 100 μ m² of 1.5 μ m sections) after 24 h *in vitro* OVA challenge. Compound 48/80 (C48/80) and dexamethasone (DEXA) treatments were performed as indicated in Materials and methods. The presence of intact and degranulated mast cells and the relative percentages are shown. Data represent mean \pm standard error of the mean of *n* = 5 rats per group.

p* < 0.05 versus saline group; *p* < 0.05 versus OVA group; n.d.: not detected.

Table 2. Cumulative data for mast cell numbers in rat distal trachea

	CTMC			MMC		
	Intact	Degranulated	% Degranulated	Intact	Degranulated	% Degranulated
Saline	8.7±0.5	0	0	1.0±0.2	0	0
C48/48	n.d.	n.d.	n.d.	0.9±0.2	0	0
OVA	0.8±0.3*	8.3±0.3*	91	0.3±0.2*	1.0±0.4*	77
C48/48+OVA	n.d.	n.d.	0	0.2±0.2	0.7±0.2	78
DEXA+OVA	6.3±0.4**	2.3±0.2**	27	0.6±0.2**	0.4±0.1**	40

Extent of intact and degranulated CTMC and MMC (as measured in 100 μm^2 of 1.5 μm sections) after 24 h *in vitro* OVA challenge. Compound 48/80 (C48/80) and dexamethasone (DEXA) treatments were performed as indicated in Materials and methods. The presence of intact and degranulated mast cells and the relative percentages are shown. Data represent mean \pm standard error of the mean of $n=5$ rats per group. * $p < 0.05$ versus saline group; ** $p < 0.05$ versus OVA group; n.d.: not detected.

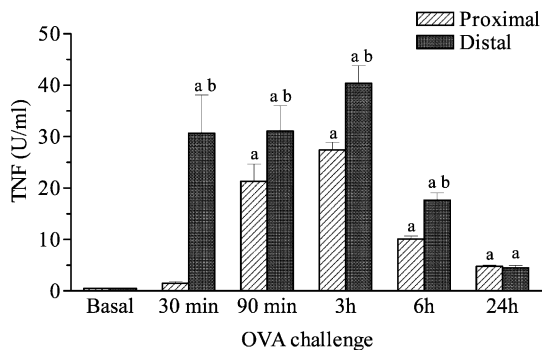


FIG. 2. Temporal profile of TNF- α activity in supernatants from proximal and distal tracheal segments after OVA challenge. Segments obtained from 14-day sensitized rats were isolated and placed in 24-well plates containing RPMI 1640 culture medium. OVA challenge was performed after 60 min, allowing for equilibrium. After the indicated time intervals, samples (100 μl) were recovered for TNF- α determination by the L-929 bioassay. The data represent mean \pm standard error of the mean from nine to 13 experiments. a, $p < 0.05$ in comparison with basal levels; b, $p < 0.05$ in comparison with the proximal segments.

in the supernatant from proximal tracheal segments were low (1.5 ± 0.3 U/ml), and reached a peak 90 min (21.3 ± 3.4 U/ml) and 3 h later (27.4 ± 1.5 U/ml). TNF- α titers fell down subsequently, remaining still approximately 10-fold above the basal levels (0.5 ± 0.01 U/ml) at 24 h (4.8 ± 0.2 U/ml).

TNF- α activity quantified in distal segments (30.7 ± 7.4 U/ml) 30 min after OVA challenge was approximately 20-fold above the amounts produced by the proximal segments, and 60 times above the respective basal values (0.5 ± 0.01 U/ml). At 3 h, the levels of TNF- α (40.4 ± 3.4 U/ml) were similar to those found at 90 min. Six hours later, the titers of TNF were reduced (17 ± 1.4 U/ml) but after 24 h these levels were 4.5 ± 0.4 U/ml (i.e. above the basal values).

Effects of compound 48/80 on the OVA-induced TNF- α release

Groups of sensitized rats were treated with compound 48/80 using a well-recognized protocol (see Materials and methods) that depleted the CTMC population from both distal and proximal trachea (Tables 1 and 2). OVA challenge induced mast cell

degranulation in the proximal and in the distal tracheal segments. Intact CTMC after *in vivo* treatment with compound 48/80 was not detected in both segments, but the number of intact MMC was not modified. The extent of MMC degranulation induced by OVA challenge was similar in both groups, treated or not with compound 48/80 (Tables 1 and 2). As observed in Fig. 3A, the proximal tracheal segments from rats pretreated with compound 48/80 released significantly higher amounts of TNF- α 90 min after OVA challenge than matched immunized and challenged rats unexposed to compound 48/80 (treated, 71.3 ± 16.2 U/ml; non-treated, 21.4 ± 2.8 U/ml). Those elevated levels of TNF- α were also found 3, 6 and 24 h after OVA challenge.

The TNF- α activity released by distal tracheal segments from rats pretreated by compound 48/80

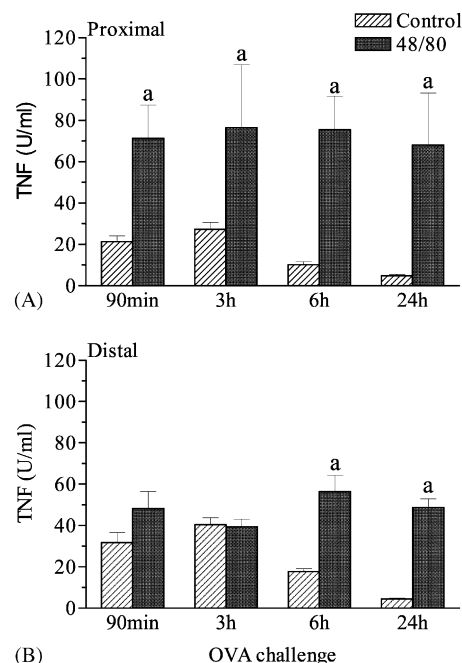


FIG. 3. Effect of *in vivo* treatment with compound 48/80 on the TNF- α activity in supernatants from proximal (A) and distal (B) tracheal segments after OVA challenge. Procedures as in Fig. 2. The control group consisted of rats treated with phosphate-buffered saline. The data represent mean \pm standard error of the mean from four to 11 experiments. a, $p < 0.05$ in comparison with the respective control segment.

at 6 h (56.4 ± 7.8 U/ml) and 24 h (48.7 ± 4.2 U/ml) after OVA challenge was significantly increased as compared with the untreated group (6 h, 17.7 ± 1.4 U/ml; 24 h, 4.5 ± 0.4 U/ml), contrasting to the levels of TNF- α at 90 min and 3 h after OVA challenge that were not affected by pretreatment with compound 48/80.

Inhibition by dexamethasone of OVA-induced TNF- α release

Fig. 4 shows the effects of dexamethasone treatment of sensitized rats on TNF- α released after *in vitro* OVA challenge of proximal (upper panel) and distal (lower panel) tracheal segments. As noted, reduced amounts of TNF- α were released by the proximal tracheal segments 90 min after OVA challenge (1.8 ± 0.3 U/ml), whereas increased levels were produced by tracheal segments from the untreated group (23.6 ± 3.7 U/ml). In addition, increased and similar levels of TNF- α were found in both, control and treated groups, 3, 6 and 24 h after OVA challenge. Dexamethasone significantly reduced the TNF- α activity produced by the distal segments (lower panel) 3 h (2.2 ± 0.7 U/ml) and 6 h (1.5 ± 0.3 U/ml) after OVA challenge, as compared with the respective control group. Dexamethasone also reduced the number of degranulated CTMC and MMC in proximal

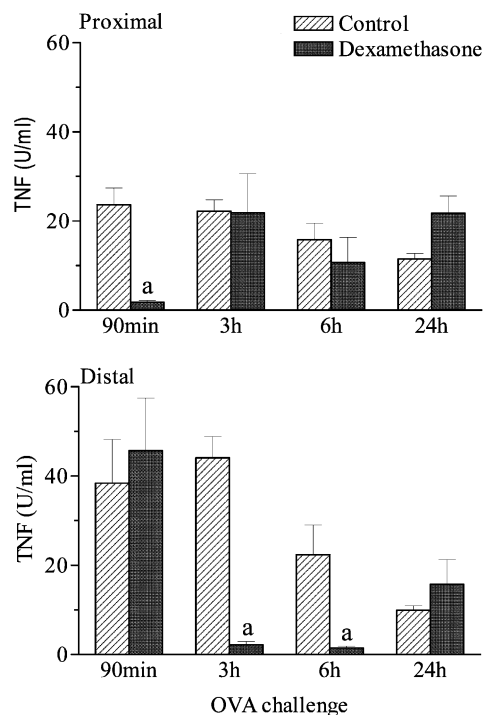


FIG. 4. Effect of *in vivo* treatment with dexamethasone (1 mg/kg intraperitoneally) on the TNF- α activity in supernatants from proximal (upper panel) and distal (lower panel) tracheal segments submitted to OVA challenge. Procedures as in Fig. 2. Data represent mean \pm standard error of the mean from five to ten experiments. a, $p < 0.05$ in comparison with the respective control segment.

tracheal segments (38% and 44%) (Table 1) and distal tracheal segments (27% and 40%) (Table 2) as compared with the untreated group

Effects of antigen challenge on eicosanoid production

Fig. 5 summarizes the results when eicosanoids were determined 30 min and 90 min after *in vitro* OVA challenge of tracheal segments isolated from sensitized rats. Fig. 5A shows that even though the basal production of PGE₂ by proximal (4.0 ± 0.4) and distal (1.8 ± 0.5) segments was significantly different, these values were not affected 30 or 90 min after OVA challenge. PGD₂ production by distal tracheal segments was significantly increased 30 min (4.0 ± 0.8) and 90 min (4.4 ± 0.8) after OVA challenge, as compared with control (unchallenged) segments (0.7 ± 0.1), whereas its production by proximal tracheal segments after OVA challenge was not different from those obtained in the unchallenged group (16.2 ± 5.6). Fig. 5C shows the significant increases of the production of LTB₄ by proximal and distal tracheal segments 30 min (proximal, 0.077 ± 0.006 ; distal, 0.2 ± 0.06) and 90 min (proximal, 0.23 ± 0.02 ; distal, 0.3 ± 0.007) after OVA challenge, as compared with the values obtained from the control unchallenged group (proximal, 0.042 ± 0.005 ; distal, 0.041 ± 0.001). Fig. 5D indicates that LTC₄ production was not affected, at any studied time point, after OVA challenge when compared with the control group.

Discussion

In this study we quantified the production of TNF- α and of eicosanoids in supernatants from proximal and distal tracheal segments of rats at distinct time intervals after *in vitro* OVA challenge, and studied the interference of dexamethasone, an effective suppressor of TNF- α production. Moreover, to study the influence of the heterogeneity of mast cell mediators on TNF- α production, we pretreated the rats with the selective CTMC-degranulating agent compound 48/80³¹ before challenging the isolated trachea with OVA.

Distal tracheal segments released rapidly significant amounts of TNF- α after challenge. This release peaked at 30 min and decayed after 6 and 24 h. By contrast, maximal values of TNF- α were released by proximal tracheal segments 90 min after OVA challenge, showing a significant difference between the tracheal segments with respect to the ability to release TNF- α . Our evidence, in conjunction with the reported release of high amounts of TNF- α after antigen challenge,^{6–8} suggests that tracheal mast cells are the major source of TNF- α produced after

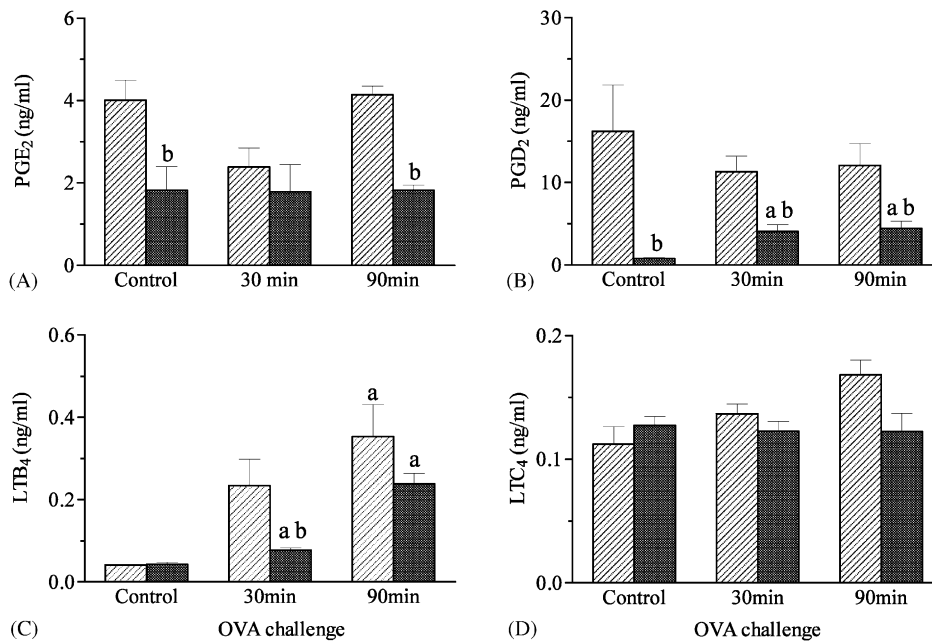


FIG. 5. Eicosanoid levels in supernatant from proximal (hatched bars) and distal (solid bars) tracheal segments after OVA challenge. Tracheal segments were isolated and placed in 24-well plates containing RPMI 1640 culture medium. After the indicated time intervals, samples (100 μ l) were recovered and PGE₂ (A), PGD₂ (B), LTB₄ (C) AND LTC₄ (D) were measured by enzyme immunoassay. Control groups consisted of tracheal segments from non-challenged tissues. Data represent mean \pm standard error of the mean from five separate experiments. Symbols over bars indicate significant differences: a, $p < 0.05$ in relation to the respective control; b, $p < 0.05$ in relation to the proximal segment.

antigenic challenge. Indeed, the increased time-dependent TNF- α activity in supernatants from tracheal segments correlated positively with mast cells degranulation caused by OVA (Tables 1 and 2).

The study of the effects of *in vitro* OVA challenge on tracheal segments is a useful addition to the standard investigations concerning the contraction of reactivity of smooth muscle caused by allergen.^{40,41} Similar procedures have been applied to study the effects of cytokines in lung fragments, as well as in isolated airways and airway smooth muscle cells in culture.^{42–45} In fact, using the distinct tracheal segments, we could analyze the effects of OVA challenge on the mast cell subtypes and their role in modulating TNF- α release.

CTMC and MMC display distinct functional activities, resulting at least in part from a different profile of the inflammatory mediators release by these cells. Mast cells, when properly activated, release rapidly preformed stores of TNF- α , followed by its synthesis and sustained release.^{46–48} Since TNF- α participates in allergic airway hyperreactivity,^{9,45} we decided to investigate whether the differential pattern of TNF- α release from the proximal and distal tracheal segments might be explained in terms of the heterogeneous mast cell population. Compound 48/80 is a selective CTMC-degranulating agent, and the dosage regimen utilized in this study confirmed its efficacy in depleting the tracheal CTMC without affecting the MMC population.³⁴ Treatment with compound 48/80 modified profoundly the temporal profile of the

release of TNF- α by both tracheal segments (see Fig. 3). Interestingly, in proximal tracheal segments, the depletion of CTMC exacerbated the release of TNF- α at all time intervals studied, whereas in distal tracheal segments the absence of CTMC affected the release of TNF- α caused by OVA challenge only after 6 h and 24 h. Since we postulated an influence of mast cell heterogeneity on the profile of the temporal release of TNF- α , we investigated the distribution of mast cells in both tracheal segments. First, we observed that OVA challenge was effective in degranulating mast cells in both segments of trachea (around 90%). Subsequently, we characterized a different pattern of distribution of MMC in tracheal segments, as compared with CTMC. The number of CTMC and of MMC in the proximal segments was similar, but in the distal segments CTMC were more numerous (Tables 1 and 2). Therefore, it is possible that the differential release of TNF- α caused by OVA challenge by proximal and by distal tracheal segment is accounted for by the presence of different populations of mast cells in these segments. Proximal and distal isolated tracheal segments show different degrees of responsiveness to allergic challenge, to serotonin, histamine, acetylcholine and to nitric oxide.^{35,49–51} Morphological analyses of both tracheas after *in vivo* treatment with compound 48/80 revealed that MMC constitute the only source of mast cells available for subsequent response to OVA. Since we observed a significant shift in release of TNF- α after OVA challenge in proximal and distal trachea, a

regulatory interaction between CTMC and MMC on the release of TNF- α after OVA challenge can be hypothesized (see Tables 1 and 2 and Fig. 3). In addition, our data suggest that TNF- α originates from MMC. In fact, MMC are the major mast cell subtype involved in increased vascular permeability induced by OVA challenge in rats.⁵²

Globally, our results allow one to hypothesize the existence of a system of regulation of the generation of TNF- α by MMC, controlled negatively by CTMC from both pools, stocked and newly formed TNF- α . Although our data do not permit one to identify the exact source of TNF- α , it is probable that proximal and distal rat tracheal cells play different roles in the regulatory mechanisms of the production of TNF- α by airway undergoing allergic response. In this context, we suggest that, after OVA challenge, CTMC and MMC are called into play distinctly, which results in a differential pattern of control of TNF- α release, where CTMC activates negatively the TNF- α release by MMC.

It is conceivable that CTMC modulates TNF- α production through the production of annexin-1,⁵³ modulating acute and even the late allergic response. In addition, TNF- α is also released by macrophages, whose functional activities are modulated by PGE₂,⁵⁴ which is released by CTMC upon challenge. This mechanism may represent a counterbalance against the inflammatory effects caused by the allergenic challenge. In addition, a local feedback loop may operate, since there is evidence that TNF- α can also decrease mast cell activation.⁵⁵ Dexamethasone reduces the mast cells degranulation caused following OVA aerolization.⁵² Since inhibition by dexamethasone of the production and release of cytokines, including TNF- α , is well documented,^{56,57} we treated rats with dexamethasone and analyzed the release of TNF- α after OVA challenge. In the proximal tracheal segments, dexamethasone partially reduced mast degranulation and prevented the release of TNF- α 90 min after OVA challenge. Glucocorticoids inhibit TNF- α gene expression mainly through post-transcriptional mechanisms.⁵⁶ Thus, our data are indicative that the TNF- α quantified at 90 min after OVA challenge is newly synthesized by mast cells, whereas at 3, 6 and 24 h TNF- α must originate from stored pools. These data agree with the view that mast cell activation via Fc γ RI accounts for rapid release of TNF- α .² Interestingly, the lack of effect of dexamethasone on the release of TNF- α in distal trachea 90 min after OVA challenge provides additional support to our view that the early TNF- α activity produced by distal segments results from the release of stocked TNF- α . By contrast, TNF- α release at 3 and 6 h might depend on its synthesis. The overall picture is that MMC and CTMC, which are equally present in the proximal segments, constitute the main sources of early (90 min) newly synthesized TNF- α , which is

sensitive to inhibition by dexamethasone. On the contrary, TNF- α released later (at 3, 6 and 24 h) should come from stocked pools and therefore is refractory to inhibition by dexamethasone. CTMC are abundant in distal segments, and may be the source for the release (90 min) of stocked TNF (also resistant to dexamethasone), whereas TNF- α released at 3 and 6 h should be newly synthesized TNF- α and therefore dexamethasone sensitive.

A complex spectrum of release of eicosanoids by proximal and distal tracheal segments was also found, whether exposed or not to antigen (Fig. 5). Neither segments released additional amounts of PGE₂ after OVA challenge. However, proximal tracheal segments spontaneously released more PGE₂ than distal segments. PGE₂ may also interfere indirectly by inhibiting mediator release from the mast cells.^{58,59} Thus, the elevated amounts of PGE₂ produced may constitute an important protective mechanism for the airways. By contrast, the proximal tracheal segments did not produce PGD₂ after challenge, whereas the distal tracheal segments produced increased amounts. Distal segments show higher amounts of CTMC (Table 2), an important cell source of prostanoids.^{32,33} In addition to its proinflammatory effects, PGD₂ induces BHR.¹⁸ Our data suggest that PGE₂ in proximal segments is constitutively released, whereas PGD₂ is released upon mast cell activation.

Both tracheal segments produced large amounts of LTB₄ upon OVA challenge (Fig. 5). LTB₄ is a major chemotactic agent, which contributes to the exacerbation of airway inflammation observed in late asthmatic response, which also stimulates vascular permeability and smooth muscle contractions.^{60,61} Its production was quantified much more intensively in the supernatants from the OVA-challenged proximal as compared with the distal tracheal segments. MMC are present in the proximal trachea and released consistently more leukotrienes than activated CTMC, which typically produce prostanoids,^{33,34} and were found in comparatively higher numbers in the distal segments. Contrasting to the distal segments, where prostanoids were found as the major eicosanoids released, the proximal tracheal segments released more LTB₄. Despite the role of cysteinyl leukotrienes on bronchial asthma,⁶² the levels of LTC₄ did not change after antigenic challenge of the trachea. The reason for such an apparent discrepancy is unclear.

In conclusion, we showed that, following OVA challenge, tracheal mast cells are activated and release TNF- α and eicosanoids. Their profile depends on the time elapsed after antigen challenge and on the tracheal segment considered. A differential fine regulation of upper airways allergic responses can thus be anticipated.

ACKNOWLEDGEMENTS. The authors are indebted to Prof. B. Boris Vargaftig of the Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo for useful discussions and revision of this manuscript. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Grants 96-09419-7, 99-06210-8, 99/04228-7). W.T.L. is a fellow researcher of Conselho Nacional de Pesquisa e Desenvolvimento (CNPq).

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Received 5 June 2003
Accepted 23 June 2003

