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Human lung mast cells release small amounts of interleukin-4 and tumour necrosis factor-α in response to stimulation by anti-IgE and stem cell factor

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Abstract

Recent reports have suggested that mast cells are capable of producing and releasing a number of pro-inflammatory cytokines. However, these studies have mainly been carried out using murine tissue culture derived mast cells and it is known that these cells differ markedly in their functional properties from isolated human mast cells. It was therefore essential to study the release of cytokines from the latter cell type. On immunological stimulation with anti-immunoglobulin E (anti-IgE) or stem cell factor (SCF), purified human lung mast cells released, within 2–10 min, small amounts of tumour necrosis factor-α (10.5 ± 2.9 pg/10^6 mast cells and 17.9 ± 7.9 pg/10^6 mast cells, respectively) and interleukin-4 (5.3 ± 2.5 pg/10^6 mast cells and 8.0 ± 3.2 pg/10^6 mast cells, respectively). After longer periods of activation (30 min–4 h), the amounts of cytokines released from stimulated cells decreased to levels which were below those of the unstimulated cells. This possible degradation of cytokines by mast cells could not be prevented by the addition of protease inhibitors.

Keywords: Mast cell; Histamine; Interleukin-4; TNF-α (tumor necrosis factor-α)

1. Introduction

Mast cells, by virtue of their location in tissues, are ideally situated to initiate local allergic responses by secreting a broad spectrum of inflammatory mediators following immunoglobulin E (IgE) receptor ($Fc_\epsilon RI$) cross-linking. The immediate phase of mast cell activation is characterised by the release of histamine and arachidonic acid metabolites, which elicit a number of effects such as bronchial smooth muscle constriction and vasodilatation which are classic components of acute allergic responses. However, chronic allergic and inflammatory reactions also involve tissue infiltration with basophils, eosinophils, lymphocytes and monocytes, which are activated by cytokines that are produced by T-helper 2 (Th2) lymphocytes and other inflammatory cells (Aggerwal and Pocsik, 1992; Del Prete, 1992). These agents include interleukin-3, -4, -5 and interleukin-10 as well as tumour necrosis factor-α (TNF-α) and granulocyte macrophage-colony stimulating factor. In recent years, most of the cytokines mentioned above have been shown to be secreted from various human and murine mast cell lines (Galli et al., 1991) although the measurement of these factors from isolated human tissue mast cells is still poorly characterised.

The main interest concerning human mast cell cytokine production has centred on interleukin-4 and TNF-α. The role of interleukin-4 in causing a switch to B-cell IgE-antibody production is well accepted (Vercelli and Geha, 1991), as is the production of interleukin-4 by activated Th2 lymphocytes (LeGros et al., 1990). However, since Th2 lymphocytes are themselves activated by interleukin-4 it has been hypothesised that the initiating stimulus to the Th2 lymphocytes may be due to mast cell-derived interleukin-4 (Bradding et al., 1992). This hypothesis appears

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to be supported by studies showing that murine mast cells release preformed stores of interleukin-4 as well as interleukin-6 which is known to stimulate B-cell proliferation and antibody production (Brown et al., 1987; Gurish et al., 1991). In the mouse, in vivo experiments showed that a rapid release of TNF-α from peritoneal mast cells resulted in neutrophil chemotaxis (Zhang et al., 1992). Further studies have also demonstrated increased endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1 expression due to the respective actions of TNF-α and interleukin-4 thus demonstrating the key role played by these cytokines in the initiation of leukocyte migration, activation and subsequent tissue invasion (Masinovsky et al., 1990; Walsh et al., 1991). Further, TNF-α stimulates the production of collagenase and prostaglandin E_2 by fibroblasts and it is thought that these effects may be linked to tissue damage found in many chronic inflammatory diseases, such as bronchial asthma.

Recent reports have shown the localisation of preformed interleukin-4 and TNF-α in human lung mast cells by immunohistochemical staining (Church and Okayama, 1995) as well as the release of interleukin-4 following immunological activation for a period of 1–6 h (Bradding et al., 1992; Tunon de Lara et al., 1994). However, in order to clarify whether mast cells are capable of rapidly (within 30 min) releasing preformed stores of these cytokines, thus implying their role as initiator cells in chronic inflammatory events, we have concentrated on this early release. Furthermore, mast cell–fibroblast interactions have been increasingly associated in tissue remodelling as well as mast cell development due to the actions of the soluble high affinity ligand for c-*kit*, stem cell factor (SCF) (Valent, 1994). We have therefore studied the effects of signalling through the Fc_ϵ receptor-1 and c-*kit* on the release of interleukin-4 and TNF-α from human lung mast cells.

2. Materials and methods

2.1. Materials

The following were purchased: HEPES, alcian blue, trypan blue (BDH Chemicals); anti-human IgE (Dako); Percoll (Pharmacia Fine Chemicals); ELISA for granulocyte macrophage-colony stimulating factor, interleukin-4, interleukin-6 and TNF-α (R&D Systems); aprotonin, bovine serum albumin, collagenase (type IA), heparin, leupeptin, *o*-phthaldialdehyde, soybean tryptase inhibitor, SCF (Sigma).

Macroscopically normal human lung parenchyma was recovered following surgery for bronchial carcinoma. The specimens were transported from the hospital in cooled (4°C) heparinized HEPES-buffered Tyrode's solution (HEPES buffer) (Pearce et al., 1985) containing bovine serum albumin (1 mg/ml), after which they were weighed and dissected free of pleural tissues, major airways and blood vessels. The samples were then chopped into fragments and washed with HEPES buffer followed by digestion with collagenase (160 U/ml) in HEPES buffer containing bovine serum albumin (1 mg/ml) as previously reported (Pearce et al., 1985).

2.2. Human lung mast cell purification

Isolated human lung mast cells (2–5% pure) were purified by counter-current centrifugal elutriation (Beckman Instruments) by a modification of the method established by Schulman et al. (1982). Typically, lung mast cells could be enriched to above 65% purity with elutriation alone, but the success of this procedure depended on the amount and type of contaminating cells which were variable between donors. Suspensions of isolated lung cells were pumped into a Beckman counter-current elutriation chamber (1025 rpm; 12 ml/min) whereupon the buffer flow rate was increased (to 25 ml/min) and cells of different size and density were collected (100 ml) at various intervals. Erythrocytes and small nucleated cells were removed at lower flow rates, whereas the majority of mast cells were collected at higher flow rates or remained in the elutriation chamber. Samples at all flow rates were collected separately, finally including cells left in the elutriation chamber, centrifuged and resuspended in HEPES buffer (2 ml) for counting with alcian blue stain. Generally, flow rates greater than 20 ml/min yielded sufficiently enriched mast cells so that these fractions could be pooled.

Elutriated human lung mast cells that were less than 60% pure were enriched further by Percoll gradient sedimentation. In this method, Percoll dilutions of 80, 60, 50 and 40% were made up from the 90% stock using HEPES buffer without added calcium. Starting with the 90% Percoll, layers of decreasing Percoll density were carefully added (5 ml) to a plastic vial. The semi-enriched mast cells were centrifuged and the pellet resuspended in the lowest (40%) Percoll concentration before layering over the 50% Percoll fraction. The tube was then centrifuged ($200 \times g$, 20 min, 4°C), after which the cells that had sedimented to the various Percoll interfaces were removed and washed several times in order to remove any remaining Percoll. Each cell fraction was counted and purified mast cells (of 65–95%) were typically found at the 50–60% and at the 60–80% Percoll interfaces (remaining contaminating cells included fibroblasts, monocytes, epithelial cells and, occasionally, macrophages). The purified cells ($92.8 \pm 1.5\%$ viable using the trypan blue exclusion test) were then diluted in the appropriate volume of HEPES buffer for the experiment.

2.3. Cytokine release experiments

Aliquots of 2.5×10^5 purified mast cells (or 3.0×10^6 total nucleated cells per ml, in experiments where purified

and unpurified human lung mast cells were compared) were suspended in HEPES buffer containing bovine serum albumin (1 mg/ml), added to Eppendorf vials and challenged with anti-IgE (1:100 dilution), SCF (10 ng/ml) or a combination of SCF and anti-IgE for various incubation times. Additionally, unstimulated cells were used as controls and were incubated in parallel with the stimulated cells. At the end of each incubation, the cells were gently resuspended and a portion (20 μl) removed and transferred to a tube containing HEPES buffer (980 μl). The cells and supernatants were then separated and assayed for histamine using a fluorometric autoanalyser (Technicon) (Pearce et al., 1985). The remaining cells were briefly placed in ice, centrifuged ($200 \times g$, 1.5 min), and the supernatants separated and frozen in dry ice. The cell pellets were resuspended in HEPES buffer containing BSA to the original volume, sonicated (3×30 s), and freeze-thawed in dry ice-methanol. These samples were then assayed by ELISA for their content of the following cytokines: interleukin-4, interleukin-6, granulocyte macrophage-colony stimulating factor and TNF-α. Standard curves for each cytokine tested were run in the same HEPES buffer used in the experiments and reference samples were included to enable us to measure concentrations down to 3 pg/ml.

Cytokine degradation was also investigated. Human lung mast cells (250 μl in HEPES buffer containing BSA, 2.5×10^5 cells per tube) were lysed as described before, followed by centrifugation ($250 \times g$, 3 min) to remove cell debris. Interleukin-4 standards were then added (250 μl) to the supernatants and the suspension was incubated (5 min and 2 h) prior to freezing for storage before assaying the interleukin-4 content.

The effects of protease inhibitors (aprotonin, leupeptin and soybean tryptase inhibitor) were also studied. All three inhibitors were added simultaneously (at 20 μg/ml) either at the start of stimulus challenge, or at the end of the incubation time. The experimental details were otherwise as already described.

3. Results

3.1. Cytokine secretion due to anti-IgE stimulation

In preliminary experiments, it was found that anti-IgE, SCF, or a combination of both stimuli induced no increase in either interleukin-4 or TNF-α release from purified human lung mast cells for periods beyond 1 h of incubation. We therefore concentrated on the possible early release of preformed stores of these cytokines. A small, yet significant, net release of both interleukin-4 and TNF-α was observed in response to anti-IgE which was maximal at 5 min and then declined towards basal levels after 1 h of stimulation (Fig. 1a). No significant net release of interleukin-6 or GM-CSF was observed in these experiments

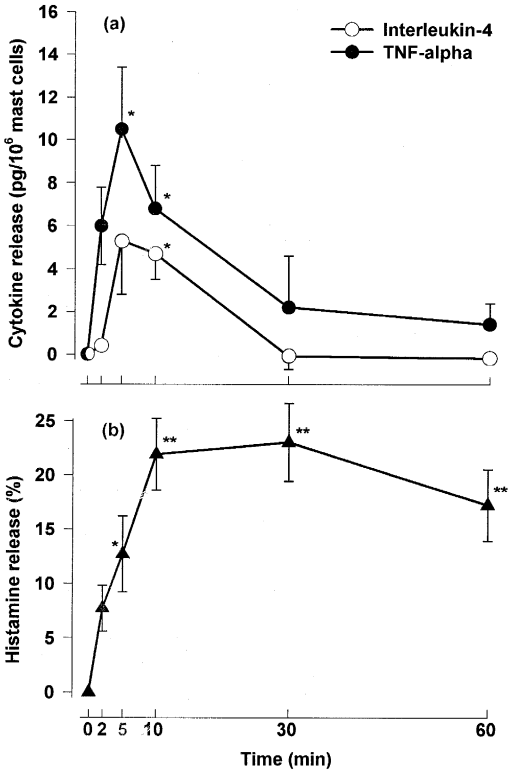

Fig. 1. Net release of interleukin-4 (open circles) and TNF-α (closed circles) (a) and histamine (b) from purified (> 65% purity) human lung mast cells stimulated with anti-IgE. All values are corrected for the spontaneous secretion and represent the mean ± S.E.M. from 3–7 experiments. Asterisks denote that the releases of cytokines or histamine are significantly (* $P < 0.05$, ** $P < 0.01$) different from the unstimulated cells.

(results not shown). In addition to the measurement of these proteins, histamine was measured in parallel (Fib. 1b) and showed that these cells were adequately responsive to anti-IgE stimulation.

Since both the net stimulated interleukin-4 and TNF-α releases were maximal within 10 min, and therefore likely to come from preformed stores rather than being newly generated, we also measured the total cytokine content of the cells. Unstimulated human lung mast cells contained 19.0 ± 6.7 pg/10^6 mast cells of TNF-α and 60.2 ± 6.3 pg/10^6 mast cells of interleukin-4 and these levels rose following incubation of the cells for 4 h to 56.9 ± 42.2 pg/10^6 mast cells for TNF-α and 103.9 ± 34.4 pg/10^6 mast cells for interleukin-4. However, although the total cytokine content of the cells was increased after longer periods of incubation, it was not further increased following stimulation of the cells with anti-IgE. Additionally, we observed a small band for TNF-α mRNA from purified human lung mast cells which was not upregulated following anti-IgE stimulation using Northern blot analysis (unpublished observations). The source of interleukin-4 and TNF-α was observed to be largely mast cell derived as

Table 1
Comparison between anti-IgE induced cytokine release from purified and unpurified human lung mast cells

Incubation time (min)	Cytokine	Purified human lung mast cells ($pg/10^6$ cells)	Unpurified human lung mast cells ($pg/10^6$ cells)	Significant difference
2	TNF-α	3.8 ± 1.0	0.4 ± 0.9	$P < 0.05$
	Interleukin-4	0.4 ± 0.1	0.5 ± 0.1	$P < 0.05$
10	TNF-α	4.5 ± 1.3	0.1 ± 0.4	$P < 0.01$
	Interleukin-4	4.2 ± 1.3	0.5 ± 0.8	$P < 0.01$

Purified (> 65%) or unpurified (2–5%) human lung mast cell preparations ($> 3 \times 10^6$ total nucleated cells per ml) were stimulated with anti-IgE (1:100 dilution) for either 2 or 10 min. All values represent the mean net releases ± S.E.M. from 3–7 experiments.

seen in terms of the net, anti-IgE stimulated, release comparing purified (65–95%) and unpurified (2–5%) human lung mast cell fractions incubated at the same cell density (Table 1).

3.2. Cytokine secretion due to SCF

The effects of SCF alone and in combination with anti-IgE were investigated on interleukin-4 and TNF-α release. As before, a steady basal release of these cytokines was observed from unstimulated mast cells with increasing incubation time. With the exception of the longest incubation time (4 h), a net release of interleukin-4 and TNF-α was observed with human lung mast cells stimulated with SCF (10 ng/ml) which was maximal for both cytokines within 10 min incubation (Table 2). The kinetics of both histamine and cytokine release due to SCF stimulation were more rapid than for human lung mast cells stimulated by anti-IgE and the net release of cytokines was more sustained for prolonged periods of incubation than for anti-IgE stimulated cells. However, the effects of SCF on interleukin-4 and TNF-α release were less consistent than those observed with anti-IgE stimulation. For both interleukin-4 and TNF-α release, there was no consistent difference between SCF alone or SCF + anti-IgE, even though the combined effects of SCF and anti-IgE led to markedly more histamine release in comparison to that of SCF alone.

Table 2
Release of TNF-α, interleukin-4 and histamine from purified human lung mast cells stimulated with anti-IgE, SCF or a combination of anti-IgE and SCF

Stimulant	TNF-α ($pg/10^6$ mast cells)	Interleukin-4 ($pg/10^6$ mast cells)	Histamine (% release)
Control	3.8 ± 1.1	2.2 ± 0.2	6.7 ± 1.6
SCF	5.7 ± 2.7	13.5 ± 6.9	10.2 ± 2.3
Anti-IgE	12.9 ± 1.9	9.1 ± 2.0	23.0 ± 3.6
Anti-IgE + SCF	10.2 ± 3.1	11.4 ± 2.9	28.8 ± 4.5

Human lung mast cells were incubated for 10 min at 37°C either with HEPES buffer alone (control) or with SCF (10 ng/ml), anti-IgE (1:100 dilution) or a combination of SCF and anti-IgE. All values represent the mean ± S.E.M. from 4 experiments.

3.3. Cytokine degradation

To investigate the possible degradation of cytokines in our studies, lysed mast cells (at 500 000 mast cells/ml) were incubated with a standard amount of purchased interleukin-4 (10, 100 pg/ml) and showed a clear degradation of this cytokine which was greatest with increased incubation time (Table 3). In contrast, no significant degradation of interleukin-4 was observed at any time point when interleukin-4 standard concentrations were incubated without mast cell lysates. Protease inhibitors were used in an attempt to inhibit any possible interleukin-4 degradation which could have arisen either during the incubation period of the experiment or during the ELISA assay. This was achieved by the addition of a mixture of leupeptin, soybean tryptase inhibitor and aprotonin (at a final concentration of 20 μg/ml for each inhibitor) which have been reported to inhibit the proteolysis of interleukin-4 (Tunon de Lara et al., 1994). These inhibitors were either added simultaneously, with the stimulus/HEPES buffer, or at the end of the experiment together with cells which were not treated by these compounds. However, in three different experiments, the addition of protease inhibitors did not lead to any increase in the amounts of interleukin-4 detected from stimulated or unstimulated mast cells. Further experiments showed that the presence or absence of bovine serum albumin in the HEPES buffer did not affect these results.

Table 3
Interleukin-4 degradation by purified human lung mast cell lysates incubated with standard concentrations of the cytokine

Incubation time	Interleukin-4 concentration (pg/ml)	Degradation (%)
5 min	10	13.8 ± 10.3
	100	25.1 ± 8.5
2 h	10	52.2 ± 5.1
	100	33.7 ± 19.1

Lysed mast cells (1×10^5) were incubated with standard amounts of interleukin-4 for either 5 min or 2 h and the amounts of interleukin-4 were determined for each tube by ELISA. Values were corrected for the control interleukin-4 concentrations that had been incubated without lysed cells and the percentage degradation calculated. Values are means ± S.E.M. for 4 experiments.

4. Discussion

These results show that human lung mast cells produce and release small amounts of interleukin-4 and TNF-α in response to immunological activation, and the first demonstration of SCF-mediated release. Although the levels measured were low, a significant difference in the net release of these cytokines was observed between purified and unpurified human lung mast cells indicating that, for short-term incubation with anti-IgE or SCF both interleukin-4 and TNF-α are mast cell derived. In addition, since the net release of these factors was observed within 10 min of stimulation, it is most likely that human lung mast cells store interleukin-4 and TNF-α as preformed mediators rather than solely synthesising them de novo after challenge. Thus, these results are in agreement with immunocytochemical staining studies that have shown the presence of preformed interleukin-4 and TNF-α in human lung mast cells (Bradding et al., 1992; Church and Okayama, 1995).

The release of preformed TNF-α has been demonstrated by Gordon and Galli (1990) in vitro in mice as well as in in vivo studies by Zhang et al. (1992) who showed that a pulse of this cytokine produced increased neutrophil influx into the mouse peritoneum. Studies on human skin mast cells by Walsh et al. (1991) also point to the early release of preformed TNF-α. The results of the present study further show the ability of human lung mast cells to release only small amounts of this cytokine.

Other studies on human lung mast cell cytokine release are scarce despite the ready availability of assay techniques. The present investigations, however, conflict with recently published data by Bradding et al. (1992) and Tunon de Lara et al. (1994) who have reported levels of interleukin-4 at concentrations up to 1000-times greater than those measured in our experiments. There are a number of potential explanations for these differences and one possible factor lies in the isolation and purification techniques used between these studies. Bradding et al. (1992) and Tunon de Lara et al. (1994) used immunomagnetic beads coupled to YB-5B8 which recognises the second domain of c-*kit* (CD 117). This positive selection procedure may provide a priming stimulus to the mast cells, although YB-5B8 binding, unlike SCF, does not appear to induce histamine release by itself (Okayama et al., 1994). However, it is not clear whether this procedure can lead to upregulation of cytokine synthesis due to possible activation of the c-*kit* receptor. In our studies, the release of interleukin-4 and TNF-α was induced either by SCF alone or in combination with anti-IgE. However, the net release of cytokine due to SCF plus anti-IgE was not profoundly different to that with anti-IgE alone. It would be interesting to see whether YB-5B8 or longer incubation with SCF can increase cytokine synthesis or alternatively prime the cells for increased cytokine release.

It is also important to consider the possibility that priming of mast cells in vivo may govern the production and release of mast cell mediators including cytokines, such as SCF, or matrix proteins. Bronchoalveolar leukocytes obtained from asthmatic subjects have been shown to secrete elevated levels of TNF-α and interferon-γ (Cembrzynska-Nowak et al., 1993). Therefore, in disease, priming effects may contribute to enhanced release of these factors from mast cells. Indeed, Tunon de Lara et al. (1994) only observed detectable amounts of interleukin-4 from 60% of patients. All donors in our experiments were elderly males (over 50 years of age) with lung cancer and the mast cell morphology and function may differ markedly from younger, particularly atopic, individuals. However, in the majority of our experiments, mast cells were adequately responsive to anti-IgE stimulation, without the need for passive sensitisation, and released equivalent amounts of histamine to that reported previously (Bradding et al., 1992; Tunon de Lara et al., 1994).

In partial agreement with previous work (Tunon de Lara et al., 1994), the present experiments also showed some degradation of interleukin-4 by mast cell lysates. However, in contrast to the former studies, this degradation could not be prevented by the use of protease inhibitors. In any event, degradation of interleukin-4 did not exceed ca. 50% when using mast cell lysates and was substantially less than this at short-time periods and higher levels of the cytokines. Therefore, even if the data were corrected for this degradation, the amounts of interleukin-4 would still fall far short of those reported above. Some evidence does exist, however, for the involvement of proteases in the regulation of cytokine concentrations. Studies by Scuderi et al. (1991) have shown that neutrophil-derived cathepsin-G inactivates TNF-α. It is thus possible that certain mast cell subpopulations release and degrade cytokines according to their location within the tissues and their protease content and by doing so play an important self-regulatory role.

The present data also differ from the situation in the basophil which, in contrast to the mast cell, is generally agreed to release interleukin-4 following immunological activation (up to 500 $pg/10^6$ basophils at 4–6 h incubation) (Brunner et al., 1993; Schroeder et al., 1994; Gibbs et al., 1996). These levels far exceed those observed in the present experiments with human lung mast cells. However, although immunologically stimulated basophils produce interleukin-4 these cells are, generally, unresponsive to non-immunological mast cell stimuli, such as SCF (Bischoff and Dahinden, 1992; Füreder et al., 1994). We have shown that SCF elicits histamine release from human lung mast cells and it has also been demonstrated that SCF can release lipid mediators from these cells (Bischoff and Dahinden, 1992). Mast cells have been implicated in various chronic inflammatory and fibrotic conditions, and fibroblasts are a major source of SCF (reviewed in: Valent, 1994). Thus, our data provide a mechanism for the participation of mast cells in both allergic inflammation and

inflammatory processes that occur independent of signalling through the high affinity IgE receptor.

Although our results demonstrate the production and release of small amounts of interleukin-4 and TNF-α due to immunologic and SCF-induced mast cell activation, the discrepancies between our data and other reports highlight the need to set methodological parameters which conserve a natural mast cell priming status and the importance of determining whether the production of these cytokines is restricted to a subpopulation of donors or mast cell types. The present published data are derived from only one centre and it is absolutely essential that independent laboratories establish the true levels of cytokines that human lung mast cells may produce under defined conditions. This is a central area of mast cell biology and, if these cells genuinely do produce significant amounts of pro-inflammatory cytokines, they may truly be assigned a key role in the orchestration of allergic inflammation.

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