Mediators of Inflammation, 9, 161–168 (2000)

GM-CSF production from human airway smooth muscle cells is potentiated by human serum

Maria B. Sukkar[1,CA], J. Margaret Hughes[1], Peter R. A. Johnson[2] and Carol L. Armour[1]

Respiratory Research Group, Departments of Pharmacy[1] and Pharmacology[2], University of Sydney, NSW 2006, Australia

[CA] Corresponding Author
Tel: +61 2 9351 3647
Fax: +61 2 9351 4391
E-mail: maria@pharm.usyd.edu.au

RECENT evidence suggests that airway smooth muscle cells (ASMC) actively participate in the airway inflammatory process in asthma. Interleukin–1β (IL–1β) and tumour necrosis factor-α (TNF-α) induce ASMC to release inflammatory mediators *in vitro*. ASMC mediator release *in vivo*, however, may be influenced by features of the allergic asthmatic phenotype. We determined whether; (1) allergic asthmatic serum (AAS) modulates ASMC mediator release in response to IL–1β and TNF-α, and (2) IL–1β/TNF-α prime ASMC to release mediators in response to AAS. IL–5 and GM-CSF were quantified by ELISA in culture supernatants of; (1) ASMC pre-incubated with either AAS, non-allergic non-asthmatic serum (NAS) or Monomed™ (a serum substitute) and subsequently stimulated with IL–1β and TNF-α and (2) ASMC stimulated with IL–1β/TNF-α and subsequently exposed to either AAS, NAS or Monomed™. IL–1β and TNF-α induced GM-CSF release in ASMC pre-incubated with AAS was not greater than that in ASMC pre-incubated with NAS or Monomed™. IL–1β and TNF-α, however, primed ASMC to release GM-CSF in response to human serum. GM-CSF production following IL-1β/TNF-α and serum exposure (AAS or NAS) was significantly greater than that following IL–1β/TNF-α and Monomed™ exposure or IL–1β/TNF-α exposure only. Whilst the potentiating effects of human serum were not specific to allergic asthma, these findings suggest that the secretory capacity of ASMC may be up-regulated during exacerbations of asthma, where there is evidence of vascular leakage.

Key words: Asthma, Airway smooth muscle, Allergy, Inflammation, Human serum, IL–1β, TNF-α, IL–5, GM-CSF

Introduction

It is now widely accepted that chronic inflammation of the airways is one of the most important factors contributing to abnormal airway smooth muscle (ASM) function in asthma. Accumulating evidence suggests that the inflammatory state of the airways induces alterations in the contractile and relaxant apparatus of ASM.[1,2] ASM also responds to it's inflammatory environment by undergoing structural changes. Inflammatory cytokines, by inducing ASMC hyperplasia and hypertrophy, contribute to thickening of the smooth muscle layer in the airway wall.[3] Increased thickness of the smooth muscle layer is a significant factor contributing to exaggerated airway narrowing in asthma.[4]

Recently, it has been demonstrated that ASMC synthesize several mediators in response to stimulation with the pro-inflammatory cytokines IL–1β and TNF-α. Stimulation of ASMC with IL–1β leads to generation of prostaglandins E_2 and I_2 consequent to the induction of cyclo-oxygenase–2 (COX–2) enzyme expression.[5,6] ASMC also synthesize GM-CSF, IL–6, IL–11 and several chemokines, including RANTES (regulated upon activation, normal T cell expressed and secreted), eotaxin and IL–8 in response to IL–1β and/or TNF-α.[7–13] In addition to inducing mediator synthesis, TNF-α also induces expression of the adhesion molecules ICAM–1 and VCAM–1 on ASMC.[14]

The ability of ASMC to synthesize mediators and express adhesion molecules in response to IL–1β and TNF-α may be critical processes contributing to both abnormal ASM function and perpetuation of the airway inflammatory process in asthma. Whilst a wide range of mediators synthesized by ASMC have been identified, these mediators may not reflect the pattern of mediator synthesis by ASMC in the allergic asthmatic state *in vivo*. Allergic asthmatic serum (AAS) is known to induce alterations in the sensitivity of ASM to contractile and relaxant agonists.[15,16] It is possible, therefore, that AAS also modulates ASMC mediator release. This possibility is further supported by the observation that IL–8, RANTES, and GM-CSF synthesis by ASMC is susceptible to regulation by the immuno-

ISSN 0962-9351 print/ISSN 1466-1861 online/00/030161-08 © 2000 Taylor & Francis Ltd

DOI: 10.1080/09629350020008673

modulatory cytokines IFN-γ, IL-4, IL-10 and IL-13.[7,10,12] The aims of this study were to examine cytokine release in response to IL-1β/TNF-α by human ASMC exposed to AAS and to investigate whether IL-1β/TNF-α prime ASMC to release cytokines in response to AAS.

Materials and methods

Materials

AAS was obtained from a single donor with severe asthma and contained >2000 IU/ml IgE. Non-allergic non-asthmatic serum (NAS) was obtained from a single non-asthmatic non-allergic donor and contained <20 IU/ml IgE. Foetal Bovine Serum (FBS) and Monomed™ were obtained from CSL Biosciences. Dulbecco's modified Eagle's Medium (DMEM), Hanks' balanced salt solution (HBSS) and all antibiotics were obtained from Gibco BRL. IL-1β and TNF-α were obtained from R&D Systems and reconstituted in sterile phosphate buffered saline containing 0.1% bovine serum albumin obtained from Sigma. Indomethacin was obtained from Sigma and reconstituted in 5% sodium bicarbonate.

Induction of cytokine release by ASMC

A description of each protocol designed to answer the study questions is provided in Table 1.

To determine whether AAS modulates cytokine stimulated mediator release from ASMC, confluent ASMC were incubated with AAS and then serum starved for either 24 or 72 h prior to stimulation with IL-1β and TNF-α or vehicle. Control treatments included cells that were incubated with either NAS or Monomed™ (Table 1: protocol 1). Following stimulation with IL-1β and TNF-α, culture supernatants were assayed for IL-5 and GM-CSF content.

In separate experiments, the influence of cell density on the response to IL-1β and TNF-α in cells pre-treated with AAS was also examined. ASMC were initially plated at a range of densities in AAS. Control cells were plated in either NAS or FBS. The cells were incubated in the various serum treatments for only 24 h to minimise the increase in cell density that would occur as a result of cellular proliferation. ASMC were serum starved for 24 h and then stimulated with IL-1β and TNF-α or vehicle (Table 1: protocol 2). Culture supernatants were assayed for IL-5 and GM-CSF content following stimulation of the cells with IL-1β and TNF-α.

To determine whether IL-1β/TNF-α prime ASMC to release mediators in response to AAS, ASMC were stimulated with IL-1β/TNF-α or vehicle and then subsequently incubated with AAS. Control cells were stimulated with IL-1β/TNF-α or vehicle and then subsequently incubated with NAS or Monomed™ (Table 1: protocol 3). Given that COX products released in response to IL-1β/TNF-α may potentially modulate the subsequent response to the various serum treatments, these experiments were also performed in the presence of the COX-1/COX-2 inhibitor, indomethacin. Indomethacin was added to the cells 1 h before and during stimulation with IL-1β/TNF-α or vehicle and the various serum treatments. Control treatments included cells where indomethacin was; (1) not present at all, (2) present during cytokine or vehicle stimulation only, and (3) present during stimulation with the various serum treatments only. Culture supernatants were assayed for IL-5 and GM-CSF content following stimulation of the cells with IL-1β/TNF-α or vehicle and following subsequent exposure to the various serum treatments.

Cell culture

Human ASM was obtained from the bronchial airways (3-6 mm internal diameter) of patients undergoing surgical resection for carcinoma or lung transplantation as previously described in detail by Hawker *et al.*[17] Briefly, smooth muscle bundles were isolated from the bronchial airways and placed in DMEM supplemented with 10% FBS and antibiotics (20 U/L of penicillin, 20 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B) and maintained in a humidified atmosphere at 37°C in air/CO_2 (95:5%, vol/vol). Fresh medium was replaced every 5 days for the first 10-20 days. Once the ASMC had grown to confluence, they were passaged and used between *passages 3–7* for experimentation. ASMC were characterised morphologically and by the presence of smooth muscle specific α-actin as determined by immunohistochemistry. All experiments were carried out in 24-well tissue culture plates in the presence of antibiotics. All treatment conditions were performed in triplicate, except the indomethacin experiments, where they were performed in duplicate.

Viable cell counts

Viable cell counts were performed for experiments in protocol 1 (Table 1). Cell counts were performed at the end of the 24 h or 72 h serum starvation period in wells separate from those used for induction of cytokine release. The adherent cells from duplicate or triplicate wells were harvested using a solution of trypsin (0.5% wt/vol in HBSS) containing 1 mM EDTA and then counted with a hemocytometer. Cell viability was determined with trypan blue exclusion; cells that stained blue were counted as non-viable.

Cytokine assays

ASMC supernatants were harvested and stored at -20°C until assayed for IL-5 and GM-CSF content by

Table 1. Description of each protocol for ASMC stimulation

Protocol	Seeding density (Cells/well)	Growth period and serum treatment (7 days (confluent) or 24 h (non-confluent))	Additional serum treatments after growth (24 h)	Serum starvation (4% Monomed) (hours)	Cytokine treatment (4% Monomed) (24 h)	Additional treatments after cytokine treatment (24 h)
1	2×10^4	7 days in 10% FBS	10% AAS or 10% NAS or 4% Monomed™	24 or 72	IL–1β+TNF-α (10 ng/ml)	
2	1, 3, 9×10^4 or 2.7×10^5	24 h in 10% FBS or 10% AAS or 10% NAS		24	IL–1β+TNF-α (10 ng/ml)	
3	1×10^4	24 h in 10% FBS		24	IL–1β or TNF-α (10 ng/ml) or IL–1β+TNF-α (5, 10, 25 ng/ml) ±indomethacin (30 μg/ml)	10% AAS or 10% NAS or 4% Monomed™±indomethacin (30 μg/ml)

All serum treatments were diluted (v/v) in Dulbecco's modified Eagle's medium (DMEM).
AAS=allergic asthmatic serum; NAS=non-allergic non-asthmatic serum; FBS=foetal bovine serum; Monomed™ is a defined serum substitute.

enzyme-linked immunosorbent assay (ELISA). Standards (rhIL-5 and rhGM-CSF) and capture/detection monoclonal antibodies were obtained from PharMingen and ELISAs were performed according to the manufacturer's directions. Purified rat anti-human GM-CSF (clone BVD2-23B6) and biotinylated rat anti-human GM-CSF (clone BVD2-21C11) were the respective capture and detection monoclonal antibodies used for quantification of GM-CSF. Purified rat anti-mouse/human IL-5 (clone TRFK5) and biotinylated rat anti-human IL-5 (clone JES1-5A10) were the respective capture and detection monoclonal antibodies used for quantification of IL-5. The limit of detection was 5 pg/ml and 10 pg/ml for the GM-CSF and IL-5 ELISAs, respectively.

Statistical analysis

In all protocols in Table 1, mean results for cytokine release were determined from triplicate or duplicate culture wells and then mean data for each treatment obtained from all cell donors. Analyses were performed on this mean data and significance was determined with ANOVA and Fisher's protected least squares difference (PLSD) test. Significance was defined as $p \leq 0.05$ in all cases.

Ethical approval

Lung samples were used with the informed consent of each patient and with the approval of the relevant Area Health Service or Hospital Ethics Committee.

Results

Cytokine release by human ASMC pre-treated with AAS prior to stimulation with IL-1β and TNF-α

Exposure of ASMC to AAS did not alter the pattern of cytokine release in response to stimulation with IL-1β and TNF-α. ASMC pre-treated with AAS prior to stimulation with IL-1β and TNF-α released similar amounts of GM-CSF as those cells pre-treated with NAS, and this was the case whether the cells were serum starved for 24 or 72 h. However, whilst the serum starvation period did not influence the response to the various serum treatments, there was a trend for increased GM-CSF production in cells starved for 24 h compared to those starved for 72 h (Fig. 1a).

Interestingly, GM-CSF release by ASMC exposed to either AAS or NAS and serum starved for 72 h was significantly less than that in their Monomed™ treated counterparts. A trend for reduced GM-CSF release following AAS or NAS exposure *vs* Monomed™ exposure was also observed in cells that were serum starved for 24 h (Fig. 1a). In addition, there was a trend for higher cell numbers in AAS or NAS treated cultures compared with their Monomed™ counterparts (Fig. 1b). Together, these observations indicated that IL-1β- and TNF-α stimulated GM-CSF release may be inversely related to cell density. This is a phenomenon we had observed previously (Fig. 2).

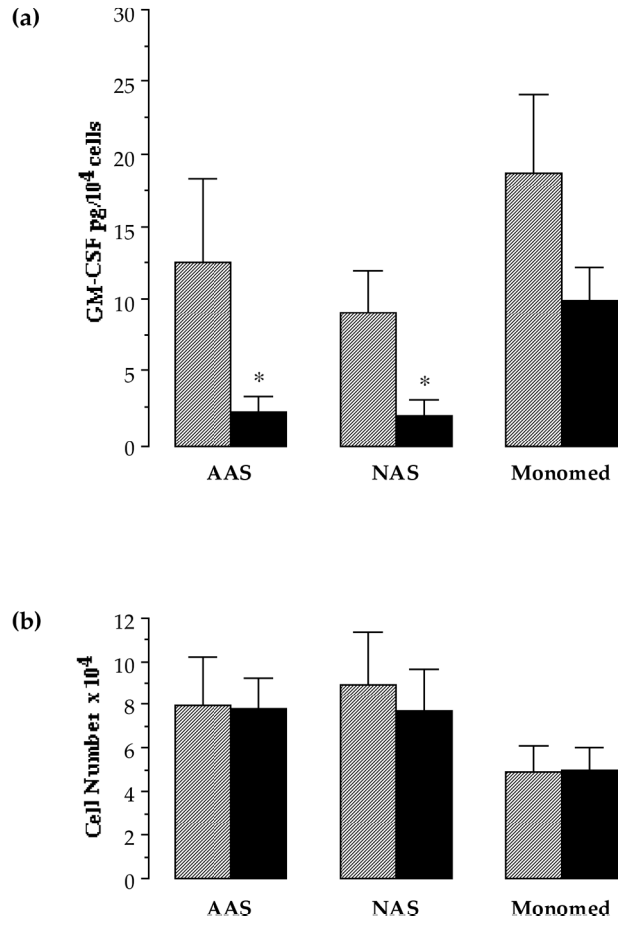

FIG. 1. (a) IL-1β and TNF-α induced GM-CSF release, and (b) human airway smooth muscle cell (ASMC) density in cultures immediately prior to stimulation with IL-1β and TNF-α. Confluent human ASMC were pre-incubated with either allergic asthmatic serum (AAS), non-allergic non-asthmatic serum (NAS) or Monomed™ (a defined serum substitute) for 24 h. Cells were serum starved for 24 h (striped bars) or 72 h (black bars) prior to stimulation with IL-1β and TNF-α (10 ng/ml each) for 24 h (Table 1: protocol 1). Bars represent means±SEM from five cell donors. *$p \leq 0.05$ compared with appropriate Monomed™ control.

ASMC did not release detectable amounts of IL-5 in response to any of the treatment conditions in protocol 1.

Influence of cell density on cytokine release by human ASMC pre-treated with AAS prior to stimulation with IL-1β and TNF-α

Given that we had evidence to suggest that GM-CSF production may be influenced by cell density, the effect of AAS on ASMC cytokine release was also examined under conditions where the influence of

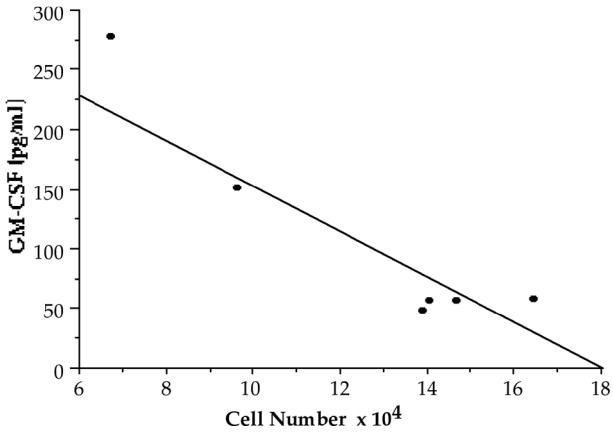

FIG. 2. Relationship between cell number and GM-CSF production. Data were obtained from human ASMC grown under various serum conditions and then stimulated with IL–1β and TNF-α (10 ng/ml) for 24 h (r=0.948; p=0.004).

cell density could be simultaneously assessed (Table 1: protocol 2). We found, that at any given initial plating density, IL-1β and TNF-α stimulated GM-CSF release in AAS treated cells was not significantly different from that in NAS and FBS treated cells (p>0.05, n=3).

However, there was a pattern of GM-CSF release that was related to cell density. This pattern was bimodal, in that GM-CSF release decreased as cell number increased at initial plating densities between 1×10^4-9×10^4 cells/well. At the highest density (2.7×10^5 cells/well), however, GM-CSF production returned back to the level that was achieved at low cell density (1×10^4 cells/well) (Fig. 3). This pattern, however, was not consistently observed under all serum conditions in all cell donors investigated.

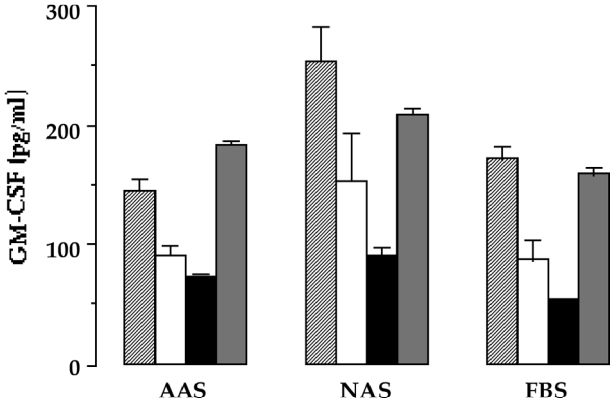

FIG. 3. IL–1β and TNF-α induced GM-CSF release in human airway smooth muscle cells plated at 1×10^4 (striped bars), 3×10^4 (white bars), 9×10^4 (black bars) or 2.7×10^5 (grey bars) cells/well in either allergic asthmatic serum (AAS), non-allergic non-asthmatic serum (NAS), or foetal bovine serum (FBS). Cells were serum starved for 24 h prior to stimulation with IL–1β and TNF-α (10 ng/ml) for 24 h (Table 1: protocol 2). Bars represent means±SD from triplicate wells of one cell donor.

ASMC did not release detectable amounts of IL-5 in response to any of the treatment conditions in protocol 2 (p>0.05, n=3).

Cytokine release by human ASMC pre-treated with IL-1β/TNF-α prior to stimulation with AAS

ASMC released significant amounts of GM-CSF in response to stimulation with IL-1β (10 ng/ml) or combined stimulation with IL-1β and TNF-α (5, 10, 25 ng/ml each) under the experimental conditions used in protocol 3. Detectable levels of GM-CSF were present in supernatants of cells stimulated with TNF-α (10 ng/ml) alone, however, these levels were not significantly different from control (Fig. 4).

IL-1β/TNF-α stimulated ASMC that were subsequently exposed to human serum (AAS or NAS), released significantly greater amounts of GM-CSF (approximately 3-4 fold greater) than cells which were stimulated with IL-1β/TNF-α but subsequently exposed to Monomed™, the serum control. Significant potentiation of GM-CSF release in response to human serum was observed following stimulation with IL-1β (10 ng/ml) or combined stimulation with IL-1β and TNF-α (5, 10, 25 ng/ml each), but not when the cells were stimulated with TNF-α alone. Furthermore, the amount of GM-CSF released by ASMC stimulated with IL-1β (10 ng/ml) or IL-1β and TNF-α (10, 25 ng/ml) and subsequently exposed to human serum was significantly greater than that released by those same cells in response to IL-1β/TNF-α stimulation alone (Fig. 4).

Indomethacin did not alter GM-CSF release by ASMC in response to stimulation with IL-1β and TNF-α (10 ng/ml). Further, the level of GM-CSF release in response to AAS, NAS or Monomed™ remained unaltered, regardless of whether indomethacin was present during stimulation with IL-1β and TNF-α only, the various serum treatments only, or both IL-1β and TNF-α and the various serum treatments.

ASMC did not release detectable amounts of IL-5 in response to any of the treatment conditions in protocol 3.

Discussion

It is now recognised that human ASMC synthesize a wide range of inflammatory mediators in response to stimulation with IL-1β and TNF-α. ASMC in the asthmatic state *in vivo*, however, are not exposed to IL-1β and TNF-α in isolation. Indeed, the capacity of ASMC to synthesise cytokines in response to IL-1β and TNF-α *in vivo* may be influenced by features of the allergic asthmatic phenotype.

GM-CSF release by human ASMC in response to IL-1β/TNF-α has previously been demonstrated.[7,8] There have been no studies which have directly measured IL-5 production by ASMC following stimulation with

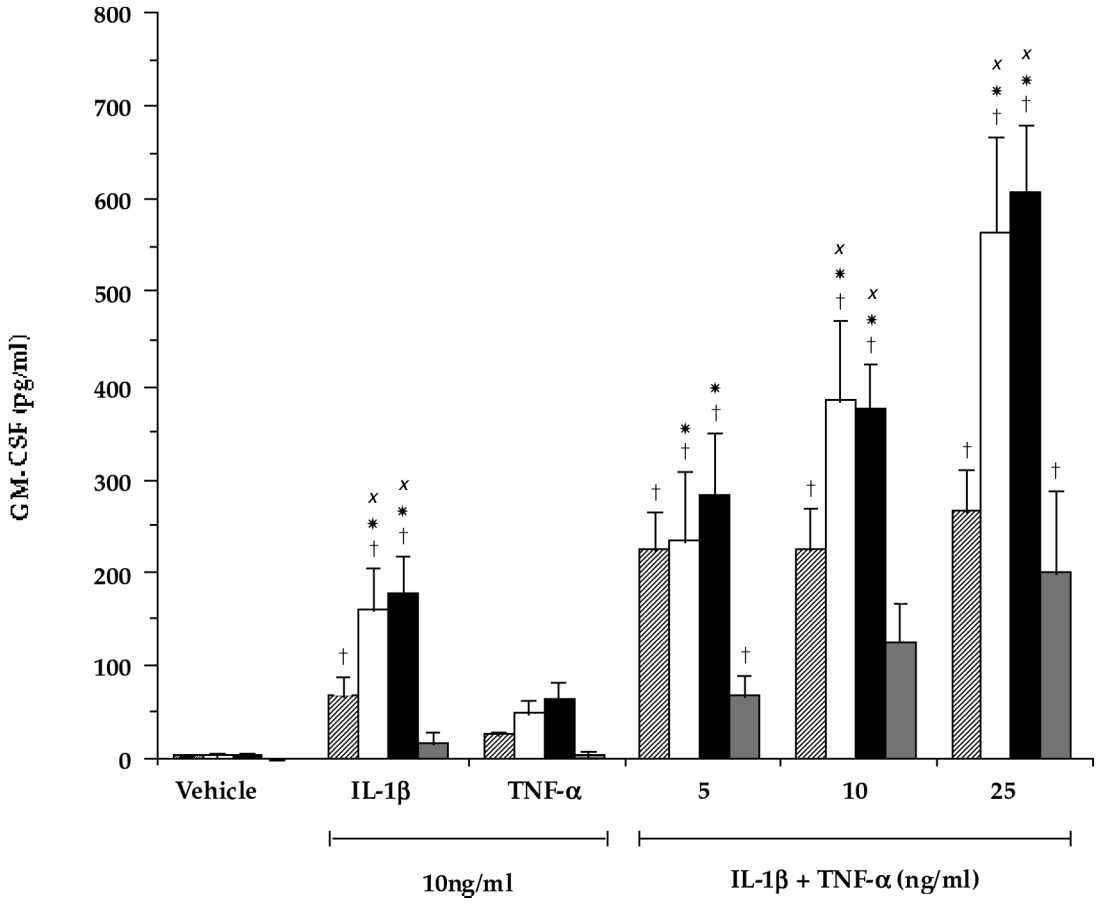

FIG. 4. GM-CSF release by human airway smooth muscle cells stimulated with IL–1β/TNF-α or vehicle for 24 h only (striped bars) or stimulated with IL–1β/TNF-α or vehicle for 24 h and subsequently exposed to either allergic asthmatic serum (AAS) (white bars), non-allergic non-asthmatic serum (NAS) (black bars) or Monomed™ (a defined serum substitute) (grey bars) for 24 h (Table 1: protocol 3). Bars represent means±SEM from four to seven cell donors. †$p \leq 0.05$ compared with cytokine vehicle control. *$p \leq 0.05$ compared with Monomed™ control (grey bars). $^{x}p \leq 0.05$ compared with cells stimulated with IL–1β and TNF-α only (striped bars).

IL–1β/TNF-α, however, a recent study by Hallsworth *et al.*[8] supports our finding that ASMC do not release IL–5 in response to IL–1β/TNF-α. These investigators demonstrated enhanced *in vitro* survival of human peripheral blood eosinophils in response to conditioned medium from IL–1β-stimulated human ASMC. The survival-enhancing activity of ASMC conditioned medium was completely inhibited in the presence of antibodies to GM-CSF, whereas antibodies to IL–5 had no effect. Our study, therefore, confirms the findings of others with regard to ASMC GM-CSF release, and extends these findings to show that 'allergic serum factors' do not influence GM-CSF release in response to IL–1β and TNF-α. Furthermore, this study provides direct evidence that stimulation of ASMC with IL–1β/TNF-α does not result in IL–5 production, even under experimental conditions which attempt to simulate the *in vivo* environment of ASMC in allergic asthma.

In examining the effect of AAS on IL–1β and TNF-α stimulated cytokine release, GM-CSF production was adjusted for cell number to account for differences in the proliferative effects of the various serum treatments (AAS, NAS, Monomed™). However, even though we had corrected for cell number, the data indicates that cell density may be a factor influencing GM-CSF production. We therefore modified the experimental protocol so that we could control for cell density between the various serum treatments at the time of stimulation with IL–1β and TNF-α (see methods: protocol 2). We found, however, that at any given cell density, GM-CSF release was similar, regardless of whether the cells were exposed to AAS, NAS or FBS. Thus, even under optimal experimental conditions, we could not demonstrate any influence of AAS on ASMC cytokine release.

From this study, it appears, that cell density and the period of serum starvation are at least two aspects of the cell culture conditions that may influence the secretory capacity of ASMC. Indeed, we observed a trend for greater GM-CSF production in cells serum starved for 24 h as opposed to 72 h under all serum conditions (Fig. 1a). Furthermore, there appeared to be an inverse relationship between GM-CSF produc-

tion and cell density. However, given that this relationship was not consistently observed suggests that other factors may influence the response of the cells to IL-1β and TNF-α. Thus, although modulation of GM-CSF release was not demonstrated under the various culture conditions used in this study, it is possible that AAS modulates ASMC cytokine release under different conditions.

In this study we also investigated whether IL-1β and TNF-α prime ASMC to release cytokines in response to AAS. We found that IL-1β and TNF-α do prime ASMC to release GM-CSF in response to AAS, however, this response is not specific to AAS. Indeed, IL-1β and TNF-α had a similar priming effect in cells that were exposed to NAS. Furthermore, GM-CSF released in response to human serum after priming with IL-1β/TNF-α (10 or 25 ng/ml each) was significantly greater than that released in response to IL-1β and TNF-α exposure only.

Recently, Hakonarson and colleagues reported up-regulation of IL-5 and IL-1β mRNA and protein synthesis in human ASMC following exposure to AAS for 24 h. Furthermore, it was shown that IL-1β release in response to AAS was due to the autocrine action of IL-5.[18,19] These investigators also reported up-regulation of GM-CSF mRNA in response to AAS.[18] In this study, we could not demonstrate GM-CSF or IL-5 release following a 24 h exposure to either AAS or NAS (see methods: protocol 3). It is important to note, however, that the amount of IL-5 detected in ASMC supernatants in the studies by Hakonarson *et al.*[18] was at the limit of detection (10 pg/ml) of our ELISA.

A possible explanation for the discrepancy in the findings between our study and that of Hakonarson and colleagues is the difference in the methods used for cell culture. In the study by Hakonarson and colleagues, human ASMC were grown to confluence in smooth muscle basal medium supplemented with 5% FBS and serum starved in unsupplemented medium for 24 h before being stimulated with AAS for a further 24 h. In our study, ASMC were seeded at low density and allowed to incubate for only 24 h in 10% FBS/DMEM, to minimise any potential influence of variations in cell density. The cells were then serum starved in Monomed™/DMEM for 24 h and exposed to 10% AAS/DMEM for a further 24 h. Apart from minor variations in the experimental protocol, the main difference is that, in our study, ASMC were non-confluent, due to the short incubation period in growth medium, whereas Hakonarson and colleagues performed their experiments on confluent cells. This may be a possible explanation for the discrepancy. Another possible explanation is that the concentration of human AAS to which the cells were exposed may have varied between the two studies. In our study, ASMC were exposed to 10% AAS/DMEM. Hakonarson and colleagues report that ASMC were incubated in AAS but do not specify the concentration of AAS that was used – if their cells were incubated in 100% AAS then this may be an important methodological difference between the two studies.

Evidence that; (1) IL-1β is released by human ASMC in response to AAS[19], and that (2) ASMC release GM-CSF in response to IL-1β, suggests that we should have observed greater potentiation of GM-CSF release in AAS *vs* NAS treated cells, as a consequence of the release and autocrine action of IL-1β. However, IL-1β also induces ASMC to release PGE_2[5,6] and, in similar structural cells, such as human lung fibroblasts, PGE_2 has been reported to inhibit IL-1β stimulated GM-CSF production.[20] Thus, it is possible, that at the time at which GM-CSF production was evaluated, inhibitory effects of PGE_2 on GM-CSF release may have masked any additional release of GM-CSF in response to AAS. However, this is unlikely, as we did not observe any modulating effects of indomethacin on serum induced potentiation of GM-CSF release.

Failure to detect any differential effects between AAS and NAS in this study is unlikely to be due to 'inactivity' of the AAS. We recently demonstrated that exposure of human ASMC to AAS, used at the same concentration and obtained from the same donor as this study, results in enhanced release of extracellular matrix proteins.[21] This suggests that the AAS used in this study is capable of modulating ASMC secretory function.

In summary, we have shown that IL-1β/TNF-α prime ASMC to release GM-CSF in response to human serum. GM-CSF promotes the survival and recruitment of airway inflammatory cells, particularly eosinophils and mast cells. Our findings suggest, that through the interaction with IL-1β/TNF-α, ASMC may play an important role in locally sustaining the airway inflammatory response in asthma.

ACKNOWLEDGEMENTS. We thank the theatre and pathology staff of the Sydney metropolitan teaching hospitals for the supply of human lung tissue and the collaborative effort of the cardiopulmonary transplant team at St Vincent's Hospital. This work was supported by the National Health and Medical Research Council of Australia. Maria Sukkar is supported by an Australian Postgraduate Award.

References

1. Black JL, Armour CL. Induction of hyperresponsiveness in human airways *in vivo* and *in vitro*. *Pulm Pharmacol* 1988: **2**: 169–78.
2. Hakonarson H, Grunstein MM. Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *Am J Respir Crit Care Med* 1998: **158**: S115–22.
3. Seow CY, Schellenberg RR, Pare PD. Structural and functional changes in the airway smooth muscle of asthmatic subjects. *Am J Respir Crit Care Med* 1998: **158**: S179–86.
4. Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 1993: **74**: 2771–81.
5. Pang L, Knox AJ. Effect of interleukin-1β, tumor necrosis factor-α and interferon-γ on the induction of cyclo-oxygenase-2 in cultured human airway smooth muscle cells. *Br J Pharmacol* 1997: **121**: 579–87.
6. Vigano T, Habib A, Hernandez A, Bonazzi A, Boraschi D, Lebret M, Cassina E, Maclouf J, Sala A, Folco G. Cyclooxygenase-2 and synthesis of PGE_2 in human bronchial smooth muscle cells. *Am J Respir Crit Care Med* 1997: **155**: 864–8.

7. Saunders MA, Mitchell JA, Seldon PM, Yacoub MH, Barnes PJ, Giembycz MA, Belvisi MG. Release of granulocyte–macrophage colony stimulating factor by human cultured airway smooth muscle cells: suppression by dexamethasone. *Br J Pharmacol* 1997: **120**: 545–6.
8. Hallsworth MP, Soh CPC, Twort CHC, Lee TH, Hirst S. Cultured human airway smooth muscle cells stimulated by interleukin–1β enhance eosinophil survival. *Am J Respir Cell Mol Biol* 1998: **19**: 910–9.
9. Elias JA, Wu Y, Zheng T, Panettiere R. Cytokine- and virus-stimulated airway smooth muscle cells produce IL–11 and other IL–6-type cytokines. *Am J Physiol (Lung Cell Mol Physiol)* 1997: **273**: L648–55.
10. John M, Hirst SJ, Jose PJ, Robichaud A, Berkman N, Witt C, Twort CHC, Barnes PJ, Chung KF. Human airway smooth muscle cells express and release RANTES in response to T helper 1 cytokines. *J Immunol* 1997: **158**: 1841–7.
11. Ghaffar O, Hamid Q, Renzi PM, Allakhverdi Z, Molet S, Hogg JC, Shore SA, Luster AD, Lamkhioued B. Constitutive and cytokine stimulated expression of eotaxin by human airway smooth muscle cells. *Am J Respir Crit Care Med* 1999: **159**: 1933–42.
12. John M, Au B, Jose PJ, Lim S, Saunders M, Barnes PJ, Mitchell JA, Belvisi MG, Chung KF. Expression and release of interleukin–8 by human airway smooth muscle cells: inhibition by Th2 cytokines and corticosteroids. *Am J Respir Cell Mol Biol* 1998: **18**: 84–90.
13. Watson ML, Grix SP, Jordan NJ, Place GA, Dodd S, Leithead J, Poll CT, Yoshimura T, Westwick J. Interleukin–8 and monocyte chemoattractant protein 1 production by cultured human airway smooth muscle cells. *Cytokine* 1998: **10**: 346–52.
14. Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* 1994: **180**: 807–16.
15. Black JL, Marthan R, Armour CL, Johnson PRA. Sensitization alters contractile responses and calcium influx in human airway smooth muscle. *J Allergy Clin Immunol* 1989: **84**: 440–7.
16. Villanove X, Marthan R, Tunon de Lara JM, Johnson PRA, Savineau J, McKay KO, Alouan LA, Armour CL, Black JL. Sensitization decreases relaxation in human isolated airways. *Am Rev Respir Dis* 1993: **148**: 107–12.
17. Hawker KM, Johnson PRA, Hughes JM, Black JL. Interleukin–4 inhibits mitogen-induced proliferation of human airway smooth muscle cells in culture. *Am J Physiol (Lung Cell Mol Physiol)* 1998: **275**: L469–77.
18. Hakonarson H, Maskeri N, Carter C, Grunstein MM. Regulation of TH1- and TH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. *J Clin Invest* 1999: **103**: 1077–87.
19. Hakonarson H, Maskeri N, Carter C, Chuang S, Grunstein MM. Autocrine interaction between IL–5 and IL–1β mediates altered responsiveness of atopic asthmatic sensitized airway smooth muscle. *J Clin Invest* 1999: **104**: 657–67.
20. Patil R, and Borch RF. Granulocyte–macrophage colony-stimulating factor expression by human fibroblasts is both up-regulated and subsequently downregulated by interleukin–1. *Blood* 1995: **85**: 80–6.
21. Johnson PRA, Underwood PA, Armour CL, Black JL. The effect of beclomethasone on atopic asthmatic serum induced production of extracellular matrix proteins by human airway smooth muscle cells. *Respirology* 1999: **4(Suppl)**: A32.

Received 9 August 2000;
accepted after revision 29 August 2000