

Pathogenesis of Bronchial Asthma

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INTRODUCTION

The pathology of bronchial asthma demonstrates a multicellular process. The airway mucosa is infiltrated with both mononuclear cells and granulocytes, of which the eosinophil is particularly prominent. In order to attempt an elucidation of the cellular biology of airways inflammation, it is important to understand both the interactions between different cells and the biology of each individual cell type. Our work has focused on the effects of monocyte and macrophage-derived cellular products on granulocytic and T cell function because of the evidence for monocyte and macrophage activation in bronchial asthma.

There is enhanced expression of $Fc\epsilon R2$ ¹⁻⁵⁾ and complement receptors⁶⁾ in peripheral blood monocytes of asthmatic patients and increased expression of complement receptors as measured by a rosetting technique, in the monocytes of asthmatic patients after allergen bronchoprovocation⁷⁾. The alveolar macrophages of patients with asthma demonstrate increased capacity to produce eicosanoid mediators and superoxide anion⁸⁾. Furthermore, these cells bear $Fc\epsilon R2$ on their surface and can be stimulated to release mediators by IgE-dependent events⁹⁻¹²⁾.

Analysis of bronchoalveolar lavage fluid of patients

with asthma after antigen challenge revealed increased amounts of β -glucuronidase whereas macrophage intracellular levels were decreased, suggesting that the macrophage secretory processes were activated by allergen^{13,14)}. Furthermore, Metzger has shown that the numbers of monocytes in the airways increase at 48 hours after antigen provocation in asthmatic patients¹⁵⁾.

INTERACTIONS BETWEEN MONOCYTES AND GRANULOCYTES

Activated peripheral blood monocytes(PBMC) generate a number of proinflammatory molecules which can influence the activity of other cell types. For instance, supernatants from activated PBMC modulate arachidonic acid metabolism by the cyclooxygenase and lipoxygenase pathway in macrophages, fibroblasts and granulocytes^{9,16-18)}. Furthermore, they activate granulocytes for certain cytotoxic functions¹⁹⁻²¹⁾. For instance LPS-stimulated monocyte supernatants were capable of augmenting the antibody-dependent cytotoxic killing of *Schistosoma mansoni* larvae by human eosinophils²²⁾. At low antibody concentrations, control medium-treated eosinophils only adhered in small numbers to the larvae and killed 2~10% of the schistosomula. In contrast, eosinophils treated with conditioned media adhered in large numbers to schistosomula and demonstrated high helmintho-toxicity(40~80%). The supernatants

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themselves were not toxic for the larvae and were shown to mediate these effects via enhanced eosinophil degranulation.

In view of the evidence implicating the role of the monocyte in the mechanisms of allergic inflammation and the suggestion that eosinophils are critical effector cells in the tissue destruction seen in bronchial asthma, we have assessed the ability of monocyte-derived molecules to modulate eosinophil function. Eosinophils cultured in the presence of 50% peripheral blood mononuclear cell-derived culture supernatants remained approximately 67% viable for 7 days. In the absence of monocyte supernatant only 15% of cells remained viable for the same period of time. The supernatants of atopic asthmatic individuals

contained more of the viability-enhancing activity than non-atopic individuals(Fig. 1)²³. On separation of the supernatant on C18 Sep-Pak columns, a major eosinophil viability-enhancing activity was eluted in the aqueous fraction, suggesting that it was a hydrophilic molecule. Neutralisation by specific antibodies indicated that the activity was completely inhibited by antiserum to granulocyte-macrophage colony-stimulating factor(GM-CSF) and was unaffected by antibodies specific to IL-3 or IL-5(Fig. 2). A second, minor viability-enhancing activity was contained in the 100% methanol fraction indicating the presence of a more hydrophobic molecule. The capacity of monocytes from atopic individuals to produce greater amounts of cytokines as compared to normal indi-

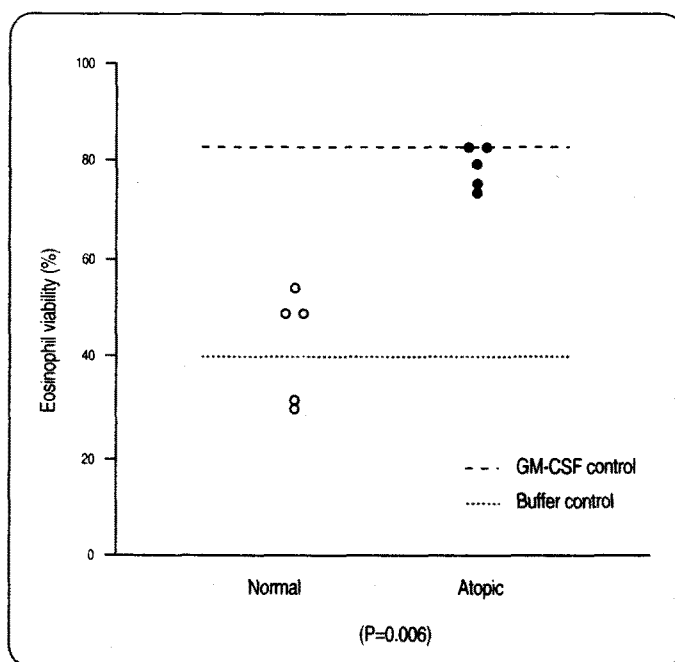


Fig. 1. Comparison of PBMC-derived culture supernatants from 5 atopic individuals with an eosinophilia of $14\% \pm 2\%$ (mean \pm SEM) with those from 5 nonatopic individuals with no eosinophilia for their capacity to enhance eosinophil viability with the same target eosinophils in the assay. The negative and positive controls are incubated with medium alone and with 1 ng/ml of rhGM-CSF, respectively.

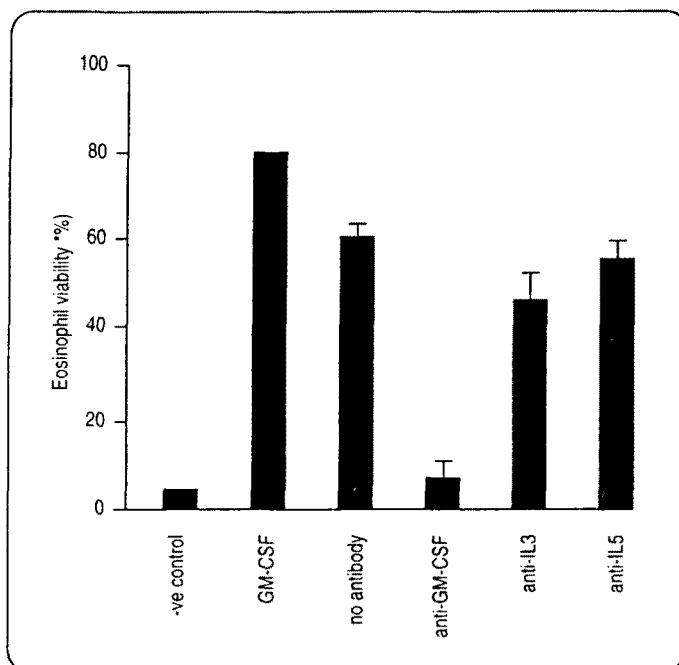


Fig. 2. The effects of different antibodies on the activity of the major eosinophil viability-enhancing activity separated by C-18 Sep-Pak fractionation. The negative control is incubation with medium alone, and the positive control is incubation with 1ng/ml of GM- CSF.

duals is not limited to GM-CSF. The monocytes of asthmatic subjects also produce approximately 3-fold more IL-1, but not TNF α and IL-8, compared to non-atopic subjects.

GM-CSF is an acidic glycoprotein with a pH of 4.5 and a molecular weight of 22,000 daltons. It is eluted from size exclusion columns with an apparent molecular weight of between 15,000 and 40,000 daltons due to variations in its glycosylation²⁴⁾ and from anion exchange between 0.10 and 0.20 M NaCl. The glycoprotein stimulates the proliferation and differentiation of normal granulocytic and monocytic stem cells¹⁰⁾ and modulates the function of mature granulocytes leading to enhancement of expression of granulocyte functional antigens 1 and 2, Mo 1, Leu M5, and C3b17,28.

GM-CSF induces histamine release from baso-

phils²⁵⁾ and enhances eosinophil survival in culture²⁶⁾. Thus, the presence of GM-CSF in the lung may precondition eosinophils for enhanced pro-inflammatory functions upon subsequent stimulation and either alone, or in concert with other cytokines, may lead to eosinophil colony formation from bone marrow progenitors. GM-CSF may play an important role in the amplification of the eosinophilic inflammation, which is characteristic of asthmatic airways.

Immunopathology of the airways

Post mortem studies of the airways of patients who died from severe asthma showed epithelial damage, plugging of the airway lumen by cellular debris, mucosal oedema, thickening of the epithelial basement membrane, intense inflammatory cell infiltration and

smooth muscle hypertrophy. These studies have suggested that the inflammation of the bronchi is an important factor in the mechanism of asthma. In order to assess the histochemical features of the cellular infiltration in asthmatic bronchi, we have obtained bronchial biopsies from 16 asthmatic patients and 6 normal subjects²⁷. In the asthmatic patients, the total numbers of macrophages infiltrating the airway

mucosa were increased. Many of these cells had the phenotypic characteristics of blood monocytes. HLA class II antigen was expressed on infiltrating cells and airway epithelial cells. In biopsies from the asthmatics there was a significant increase in activated eosinophils, but not in neutrophils.

There was also a significant increase in the numbers of T lymphocytes in the asthmatic subjects, with

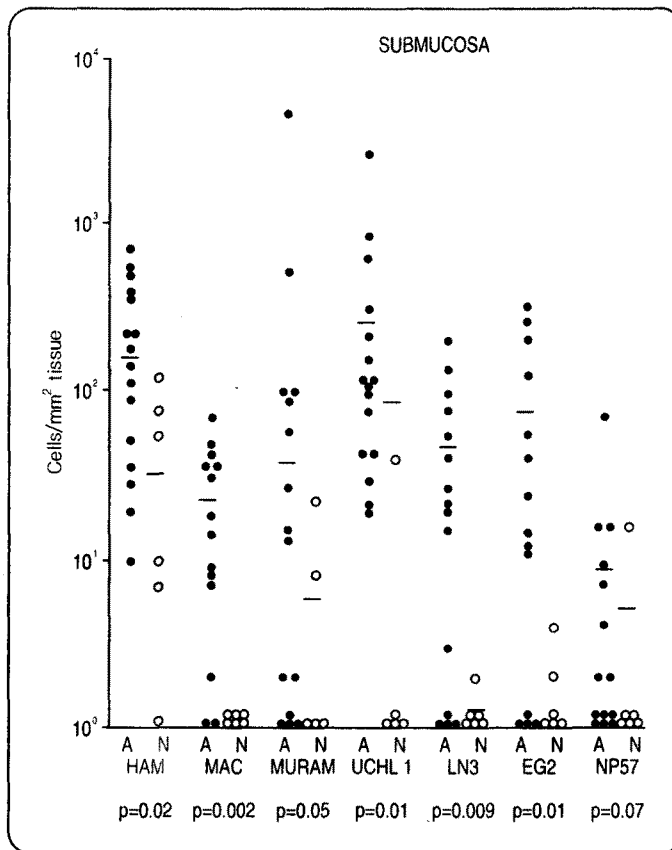


Fig. 3. Individual cell counts in the submucosa for each of the antibodies used on biopsies obtained from asthmatic(A) and from normal subjects(N). HAM = HAM 56: pan-macrophage marker; MAC387 = monoclonal anti-monocyte monoclonal antibody; MURAM = polyclonal anti-muramidase(lysozyme); UCHL1 = anti-memory T- cell monoclonal antibody; LN3 = anti-HLA-DR monoclonal antibody; EG2 = anti- eosinophil cationic protein monoclonal antibody; NP57 = anti-neutrophil elastase monoclonal antibody. The p values given were obtained using the Mann-Whitney U test.

very few B lymphocytes detected(Fig. 3).

These results suggest that lung macrophages may have a role to play in the mechanisms of the chronic immune-mediated inflammatory response seen in the airway mucosa of asthmatic patients. Furthermore, the findings suggest that there is a heterogeneity of macrophage subpopulations in the asthmatic airway. The existence of phenotypically and functionally distinct macrophage subpopulations in human airways is supported by data from Spiteri and Poulter. Using the monoclonal antibodies RFD1 and RFD7, they have defined a population of macrophages(RFD1+, D7-) which strongly support T cell proliferation, whereas RFD1+, D7+ macrophages suppress T cell responses²⁸⁻³⁰. The presence of functionally distinct macrophage subsets is further supported by observations in the rat³¹.

Using polyclonal and monoclonal antibodies to GM-CSF for immunohistochemistry of airway biopsies obtained by fiberoptic bronchoscopy, we have shown that there are 7-fold greater numbers of GM-CSF staining cells in the bronchial mucosa of asthmatic patients than in that of normal subjects. Approximately two-thirds of cells staining for GM-CSF are macrophages and approximately three-quarters of macrophages stain positively for GM-CSF. In addition to the staining of mucosal inflammatory cells for GM-CSF, the epithelial cell stains very strongly for this cytokine. The extent of staining, as quantitated by hue saturation-intensity colour image analysis, indicated that asthmatic airway epithelial cells stained more strongly with a polyclonal or monoclonal antibody to GM-CSF than that of normal subjects($p=0.013$ and $p=0.003$ respectively). Administration of inhaled beclomethasone dipropionate 1,000 μ g daily for 8 weeks resulted in a significant reduction in GM-CSF expression in the airway epithelium in patients given corticosteroids as compared to those given placebo. There was a correlation

between the suppression of GM-CSF staining by inhaled corticosteroids and the increase in FEV₁ and decreased carbachol responsiveness.

The present findings are consistent with *in vitro* data demonstrating the production of GM-CSF, IL-1, IL-6 and IL-8 by cultured airway epithelial cells in the presence and absence of stimulation by IL-1 or TNF^{32,33}, GM-CSF primes macrophages for enhanced IL-1 production and IL-1 activates epithelial cells which produce GM-CSF. Thus the macrophage/IL-1/epithelial cell/GM-CSF interaction may form the basis of a potent pro-inflammatory amplification mechanism.

INTERACTIONS BETWEEN ALVEOLAR MACROPHAGES(AM) AND GRANULOCYTES

In view of the evidence that AM secrete molecules which can influence the functions of inflammatory granulocytes³⁴ and the compelling evidence for the participation of eosinophils in airways inflammation, we have studied the interactions of AM and eosinophils in asthmatic subjects.

Eosinophils incubated with supernatants from AM from asthmatic patients, followed by stimulation with A23187, resulted in enhancement of the capacity of eosinophils to secrete LTC₄(mean enhancement 169 \pm 37%, $n=31$)³⁵. AM supernatants derived from normal individuals had no enhancing effects when compared with culture medium. Enhancement was maximal when eosinophils were preincubated with a 1:6 dilution of AM supernatants for 5 minutes at 37 $^{\circ}$ C and then stimulated with 5 μ M A23187 for 5 minutes at 37 $^{\circ}$ C. There was an inverse correlation between the % enhancement and the baseline LTC₄ production($r=-0.48$, $p<0.01$). Partial purification of the enhancing activity by HPLC on a TSK G3000 SW column revealed that it had a molecular size of approximately

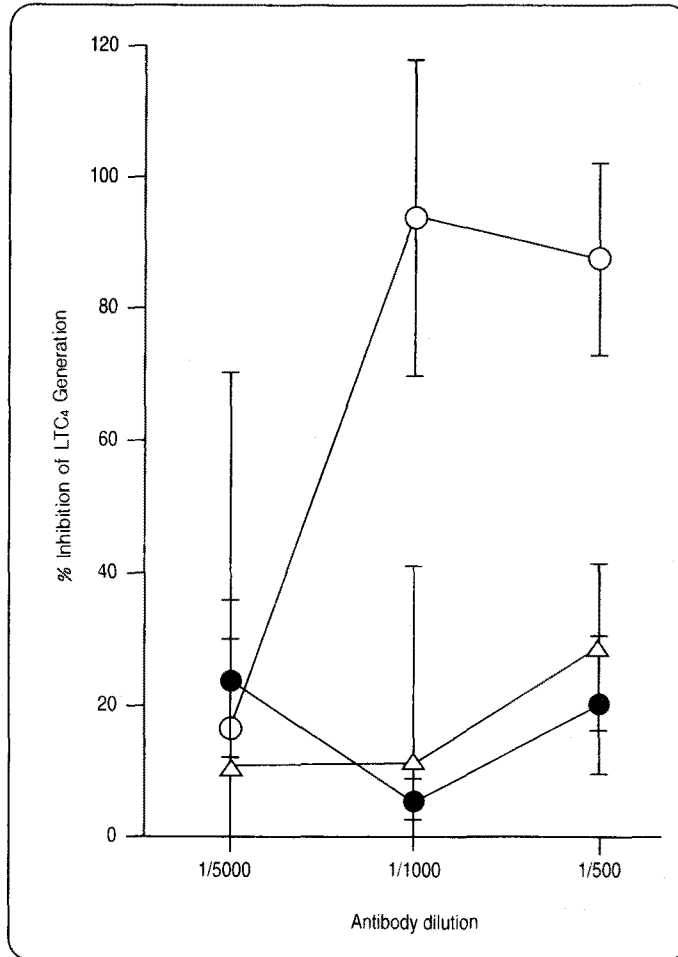


Fig. 4. The antibodies used were directed against GM-CSF(○), G-CSF(●) or, as a control, serum obtained from animals prior to immunisation with GM-CSF was used(△). Each point represents mean \pm SEM(n=4) except for anti-GM-CSF at 1:5000(n=2) and anti G-CSF at 1:5,000(n=2).

30,000 daltons and the activity was eluted in two consistent peaks at 0.17M and 0.2M NaCl from anion exchange HPLC(TSK DEAE 5 PW column, pH 7.4). The activities were distinct from IL-1 and TNF. The major activity which eluted at 0.2M NaCl was further resolved by RP-HPLC on a C18 Spherisorb column with a slope gradient of 0 to 100% acetonitrile. A single peak of activity was eluted at 41% acetonitrile. The activity was inhibited by trypsin

digestion and heat, and was neutralized by incubation with specific antibodies to human GM- CSF(Fig. 4). This suggested that the major active component is identical or closely related to GM-CSF. This finding is supported by the observation that pretreatment of eosinophils with recombinant GM- CSF primed the cells for enhanced LTC₄ generation following stimulation with A23187 or unopsonized zymosan. The priming may be mediated by augmented release

of arachidonic acid from membrane phospholipids^{16,17} caused by enhancement of the rate of membrane depolarisation induced by subsequent stimulation³⁶. The enhancement was dose-dependent and the maximal enhancement of LTC₄ occurred at 1 ng/ml GM-CSF for A23187 stimulation and at 10 ng/ml GM-CSF for stimulation with unopsonized zymosan.

ANTIGEN PRESENTATION TO T CELLS

GM-CSF and interferon- γ (IFN- γ) increase HLA class II molecule expression and enhance antigen presentation. Since GM-CSF production is increased in asthma the capacities of peripheral blood monocytes and macrophages obtained by bronchoalveolar lavage to present antigens to autologous T cells *in vitro* have been assessed in normal and atopic asthmatic individuals³⁷. Monocytes presented the recall antigens, tuberculin protein purified derivative (PPD) and streptokinase- streptodornase(SKSD), to T cells better than macrophages in most of the individuals, as assessed by [³H]-thymidine uptake. This may be due to the recognised inhibitory activity of macrophages on T cell proliferation³⁸. In the asthmatic group, a number of individuals demonstrated greatly augmented macrophage antigen presenting capability relative to that of peripheral blood monocytes³⁷. Aibas has previously suggested that airway macrophages of asthmatic patients have a less inhibitory activity on T cell proliferation than normal subjects³⁹. Since monocytes present antigen better than macrophages and have less inhibitory activity on T cell proliferation, the increased efficacy of airway macrophages to activate T cells in asthmatic patients is consistent with the presence of an immature macrophage population in the airways. The possibility that the enhanced antigen presenting capability of the macrophage population seen in certain patients with

bronchial asthma may be due to the presence of increased numbers or the function of dendritic cells has not yet been evaluated³¹.

There was a correlation between bronchoalveolar lavage lymphocytosis and the relative ability of the alveolar macrophages to present recall antigens as compared to monocytes in asthmatic, but not in normal subjects³⁷(Fig. 5). The finding that airway lymphocytosis correlates with accessory cell function can be explained in at least two ways. In the first place, the presence of an alveolar lavage macrophage population, with abnormally enhanced accessory cell function, may be responsible for the infiltration of increased numbers of activated T cells in asthmatic airways. The T cells in asthmatic airways are predominantly of the Th₂ sub-type with a cytokine profile characterized by IL-4 and IL-5. Thus, macrophages may determine the proliferation or recruitment of Th₂ cells in asthmatic airways. Much evidence suggests that lymphocyte localisation to the lung may be controlled by signals such as locally produced cytokines in the lung, synthesized by both the lung parenchyma and pre-existing leukocytes⁴⁰. Alternatively, the elaboration of a hitherto uncharacterised factor from lung lymphocytes may alter the function of resident macrophages. Evidence exists for the recruitment of distinct lymphocyte subsets to the lungs, with over-representation of certain T cell receptor arrangements⁴¹. Furthermore, recently-activated lymphocytes also preferentially migrate into the bronchoalveolar lavage compartment of the lung and have been found in bronchial biopsies of asthmatic patients^{42,43}.

We have failed to find a similar correlation between the lavage lymphocytosis and accessory cell function for the presentation of PPD and SKSD in 14 patients with sarcoidosis⁴⁴, a condition which is also characterised by enhanced alveolar macrophage accessory cell function and pulmonary lymphocyte

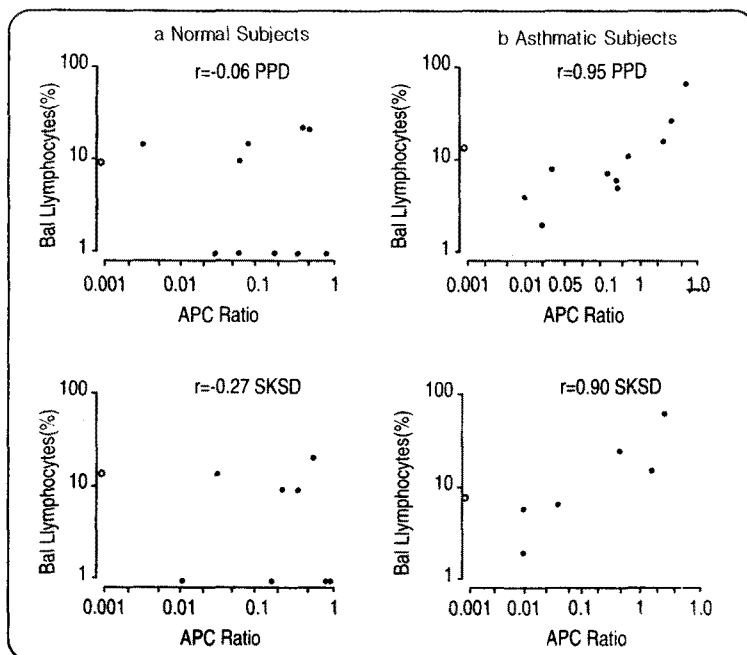


Fig. 5. Correlation between percentage lymphocytosis in bronchoalveolar lavage(BAL) and the antigen presenting cell(APC) ratio in normal(n) and in asthmatic(b) subjects for purified protein derivative(PPD) and streptokinase-streptodornase (SKSD). The APC ratio is the ratio of macrophage:monocyte antigen presenting capability. Each point is an individual subject, and the open circles indicate an APC ratio of zero. The correlation coefficient(r) was calculated by linear regression analysis.

infiltrates. Thus, whilst it is possible that a local subset of activated T cells may be responsible for enhancing alveolar macrophage function, this is seen only in asthma and not in sarcoidosis. Whether the relationship we describe between the alveolar macrophage accessory cell function in lymphocytes in asthmatic individuals can be explained by infiltration into the asthmatic lung of functionally distinct subsets of site- and allergen-specific CD4 positive cells, with a specific pattern of cytokine release⁴⁵, remains to be determined.

REGULATION OF GM-CSF

In view of the evidence for up-regulation of GM-

CSF gene expression in asthma, it becomes pertinent to address the regulation of GM-CSF gene expression. Studies of the promoter region of GM-CSF with reporter gene plasmids have identified two important *cis* acting sequences, a 10 bp GAGATTCCAC positioned at -91 to -101 bp from the transcription initiation site and a 19 bp conserved region TCACCATTA ATCATTTCCT from -34 to -52 bp^{46,47}. The 10 bp fragment belongs to the lymphokine consensus sequence GRGRTTYCAY (where R is A or G and Y is T or C) which is common in mouse and human IL-2, IL-4, GM-CSF and hIFN- γ and which binds a protein present in cells synthesising GM-CSF⁴⁸. The 19 bp sequence belongs to a region which is 100% conserved between mouse and human GM-CSF genes

and shares an octanucleotide with IFN- γ . Apart from IFN- γ , this region is common only with IL-5, suggesting that in some tissues or circumstances the two genes may be expressed in concert.

The function of the -34 to -52 bp element is tissue-specific. It has no effect in non-producers of GM-CSF(COS cells and adenovirus-transformed human kidney cell line 293) but is active in Jurkat cells and endothelial cells after activation with PHA and PMA⁴⁹). An important breakthrough has been made recently: In human fibroblasts, PMA activation leads to increased GM-CSF transcription under the control of this element while TNF α and IL-1 activation of the GM-CSF expression is regulated post-transcriptionally via the 3'-untranslated region(UTR) of the

mRNA⁵⁰). There is evidence that the AU-rich sequences of the 3'-UTR also act in a tissue-specific manner^{51~53}). The basal expression of GM-CSF in quiescent cells has not been detected but specific as well as nonspecific activation causes its expression in purified T cells, monocytes, macrophages, fibroblasts, endothelial cells and a number of cell lines. Mouse peritoneal macrophages express GM-CSF after stimulation with a number of nonspecific inflammatory agents(LPS, FCS), phagocytosis and adherence in the presence of fibronectin *in vitro*. Transcription starts between 1 and 2 hours after activation, and reaches maximum between 2 and 8 hours. Activation of GM-CSF transcription and mRNA accumulation is prevented by dexamethasone and IFN- γ ⁵⁴). In con-

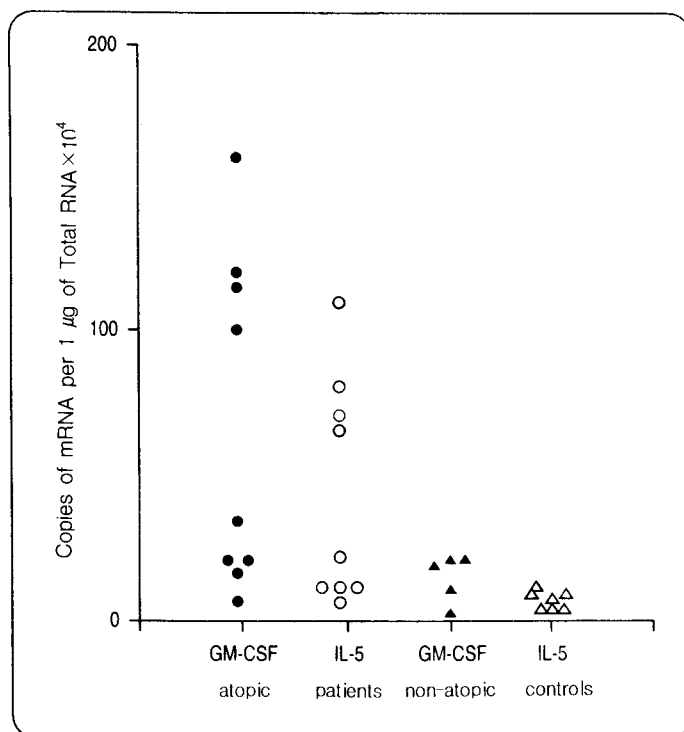


Fig. 6. IL-5 and GM-CSF mRNA(copies/mg total RNA) in peripheral blood mononuclear cells(PBMC) of atopic and non-atopic subjects after activation with 5ng/ml PMA for 20 hours. Two of the non-atopic subjects were monitored only for IL-5 mRNA.

trast, cyclosporin A suppresses the activation of IL-2, IL-3 and IFN- γ in mouse spleen cells and thymoma cells but fails to inhibit GM-CSF⁵⁵⁾.

Stimulation of a murine mastocyte line with PMA and/or ionophore shows that GM-CSF is regulated at transcriptional and post-transcriptional levels while IL-3 is regulated at the post-transcriptional level only⁵⁶⁾. Both cytokines accumulate during the first hour of activation. A similar rapid accumulation of IL-3 and GM-CSF mRNAs was observed when bone marrow mast cells were activated by the binding of IgE to the high-affinity IgE receptor. Maximum mRNA levels were observed after 30 minutes and were not detectable after 2 hours⁵⁷⁾. When mouse mast cell lines were activated with ionophore and/or via the high affinity receptor of IgE (Fc ϵ R1), mRNAs of IL-3, IL-4 and IL-5 accumulated after 2 hours⁵⁸⁾. Both studies show that while GM-CSF can be activated by PMA or ionophore alone, IL-3 requires

calcium ionophore.

In T cells both cytokines are expressed by activation via T cell antigen receptors and by non-specific mitogens, but they can also be induced by PMA alone. When TH2 type mouse cell lines were activated with immobilised anti-CD3 they expressed c-myc, c-myb, IL-4, IL-5 and GM-CSF; when activated with IL-2 they expressed c-myc, c-myb and IL-5, and when activated with IL-4 they expressed IL-5 only. Cyclosporin A inhibited IL-4 but not IL-5 and cyclo-heximide inhibited IL-5 but not IL-4⁵⁹⁾. IL-5 mRNA was detected after 6 hours and increased for 24 hours. Thus the expression of cytokine genes may be stimulus-specific and the regulation may be specific for the cytokine.

We have studied the levels of IL-5 and GM-CSF mRNA in peripheral blood mononuclear cells(PBMC) of a number of atopic and non-atopic individuals. In order to obtain quantitative results we have developed

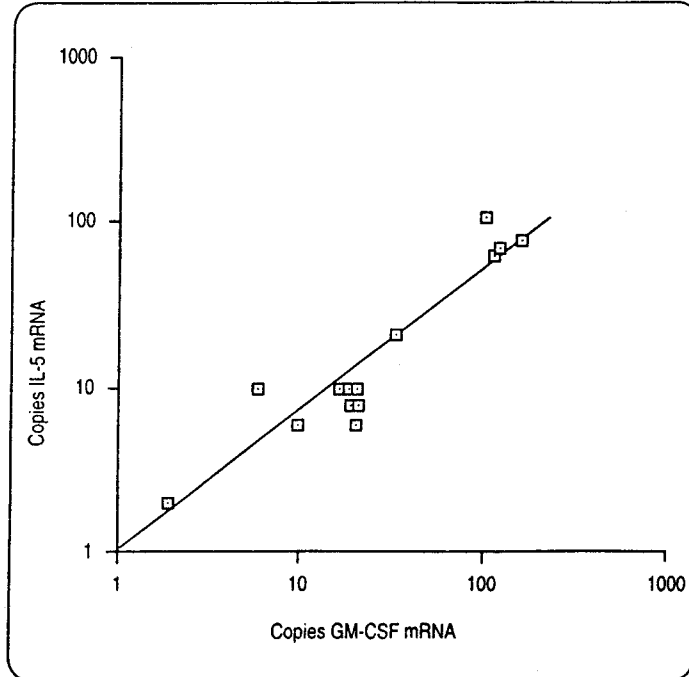


Fig. 7. Copies of IL-5 mRNA vs. copies of GM-CSF mRNA in PBMC of all the individuals studied.

a quantitative polymerase chain reaction(PCR) analysis of the corresponding cDNAs⁶⁰. Activation of the cells with PMA(5ng/ml) for 20 hours led to expression of high levels of IL-5 and GM-CSF mRNA. There was a marked difference between the levels of mRNA for both genes in some atopic patients as compared to non-atopic control subjects, suggesting that in some atopic patients IL-5 and GM-CSF may contribute to eosinophilia(Fig. 6). There was a significant correlation between the levels of IL-5 and GM-CSF mRNA in activated cells(Fig. 7). Thus high IL-5 producers are also high GM-CSF producers. Transcription of IL-5 and GM-CSF started about 3 hours after activation and increased well over

15 hours after which time it remained stable for another 24 hours. There was a small but significant lag period of 3 hours between the kinetics of GM-CSF and IL-5 mRNA levels suggesting that the transcription of the two genes may be regulated by different mechanisms and that expression of GM-CSF may be required for the increase in the IL-5 mRNA level(Fig. 8). In order to test the latter possibility we incubated mononuclear cells in the presence of (i) rhGM-CSF for 3 and 20 hours, (ii) rhGM-CSF and PMA for 2 and 20 hours, (iii) anti-GM-CSF monoclonal antibody and PMA for 20 hours and (iv) PMA alone. IL-5 mRNA was not detected for up to 20 hours with rhGM-CSF alone. After 2 hours of

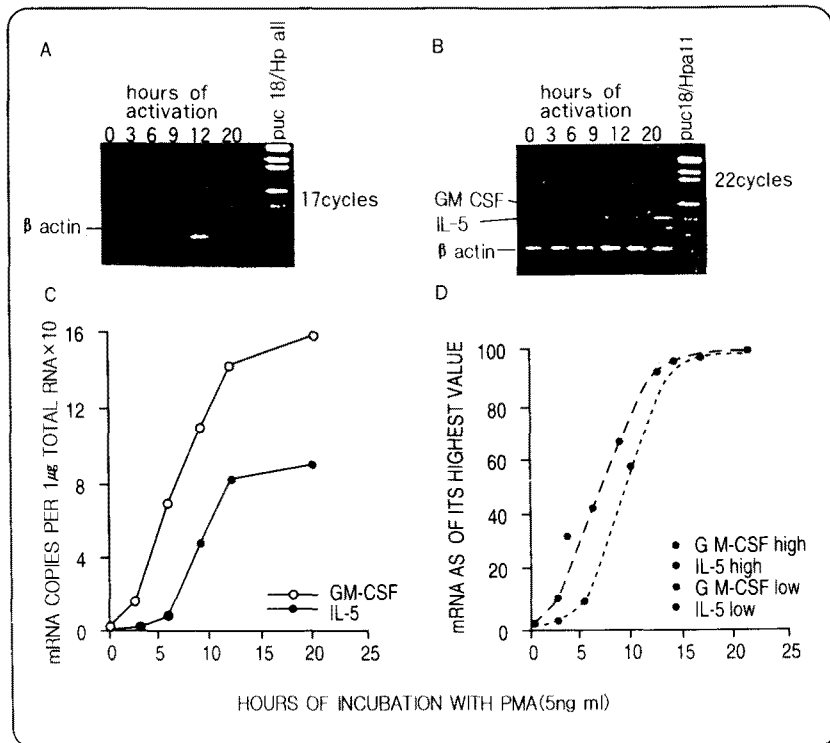


Fig. 8. A/B/C/D. Agarose gels of PCR amplified mRNA of a high IL-5 and GM-CSF producer after activation with 5 ng/ml PMA for 0,3,6,12 and 20 hours. PCR has been carried out with primers for the three mRNAs simultaneously. a = after 17 cycles, b = after 22 cycles. c = time course IL-5 and GM-CSF mRNA (copies/mg total RNA) calculated from b. d = time course of IL-5 and GM-CSF mRNA as percentages of the maximum level for a high- and a low-IL-5 producer.

activation with PMA the presence of rhGM-CSF did not result in the appearance of IL-5 mRNA and the GM-CSF mRNA level was as weak as it was after activation with PMA alone. Finally, the presence of anti-GM-CSF antiserum did not inhibit PMA activation of either.

Our results, taken together with previously published data suggest that the time course of activation of GM-CSF and IL-5 gene transcription is tissue-specific with different time courses of expression. Regulation of GM-CSF and IL-5 gene transcription always required an intermediate step involving protein synthesis. This suggests that they are expressed under the control of a, so far unidentified, gene(s).

In order to study the regulation of GM-CSF at the genomic level, we have turned to the techniques of molecular biology. We have recently found a novel regulatory element in the promoter of the GM-CSF gene⁶¹. It contains two symmetrically nested inverted repeats(-192CTTGGAAAGGTTTCATTAATGAAAAC CCCCAG -161). In transfection assays using the human GM-CSF promoter, this element has a strong positive effect on the expression of a reporter gene by the human T cell line Jurkat J6 upon stimulation with phorbol dibutyrate and ionomycin or anti-CD3. This element also acts as an enhancer when inserted into a minimal promoter vector. In DNA band-retardation assays, this sequence produces 6 specific bands which involve one or other of the inverted repeats. We have also shown that a DNA-protein complex can be formed involving both repeats and probably more than one protein. The external inverted repeat contains a core sequence CTTGG...CCAAG which is also present in the promoters of several other T cell-expressed human cytokines (interleukins 4,5 and 13). The corresponding elements in GM-CSF and IL-5 promoters compete for the same proteins in band-retardation assay. The palindromic elements in these

genes are larger than the core sequence, suggesting that some of the interacting proteins may be different for different genes. Considering the strong positive regulatory effect and their presence in several T cell-expressed cytokine genes, these elements may be involved in the coordinated expression of these cytokines in T helper cells.

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