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Expression of interleukin-5 and granulocyte–macrophage colony-stimulating factor in human peripheral blood mononuclear cells after activation with phorbol myristate acetate

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SUMMARY

The expression of interleukin-5 (IL-5) and granulocyte–macrophage colony-stimulating factor (GM-CSF) was studied in peripheral blood mononuclear cells (PBMC) from atopic and non-atopic subjects after activation with phorbol 12-myristate 13-acetate (PMA). The levels of IL-5 and GM-CSF mRNA were monitored by quantitative polymerase chain reaction (PCR). IL-5 and GM-CSF mRNA was undetectable in quiescent cells. Following PMA stimulation, some atopic patients showed considerably higher levels of IL-5 and GM-CSF mRNA expression than the non-atopic subjects, and there was a significant correlation between the levels of these two cytokines. It was found that activation of IL-5 expression in PBMC requires protein synthesis as does activation of GM-CSF expression, and that PMA is only required during the first few hours of activation. The kinetics of activation indicated that the level of both mRNA increased over 15 hr and remained constant for another 20 hr. The accumulation of IL-5 mRNA lagged about 3 hr behind GM-CSF mRNA accumulation, suggesting that the expression of these two genes is regulated separately. However, GM-CSF expression was not required for IL-5 activation.

INTRODUCTION

T cells or T-cell-secreted factors play an important role in regulating eosinophilia. *In vitro* experiments have shown that eosinophil differentiation and survival can be induced or enhanced by interleukins-3 and -5 (IL-3, IL-5) and granulocyte–Macrophage colony-stimulating factor (GM-CSF).[1–6] Of these three haemopoietic cytokines, only IL-5 is specific for eosinophils. A recent study of transgenic mice expressing IL-5 under the control of the dominant control region of the human CD-2 gene has shown that overexpression of IL-5 is sufficient for development of eosinophilia *in vivo*.[7] Whilst the function of these cytokines has been extensively investigated, the available data regarding their expression concern mainly IL-3 and GM-CSF, and very little is known about IL-5.

Basal expression of IL-5 has not been detected in quiescent cells, but it is expressed by peripheral blood mononuclear cells (PBMC), purified T cells and T-cell clones after activation with immobilized anti-CD3, phorbol 12-myristate 13-acetate (PMA), phytohaemagglutinin (PHA), IL-2 and IL-4.[8–10] IL-5 is expressed at a higher level in PBMC isolated from patients with parasite-induced eosinophilia,[10] Kimura's disease[9] and idiopathic hypereosinophilic syndrome.[6] In this paper we report the level and time-course of accumulation of IL-5 and GM-CSF mRNA in PBMC from atopic and non-atopic individuals after activation with PMA, utilizing quantitative PCR amplification of the corresponding cDNA.

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MATERIALS AND METHODS

Materials

PMA was purchased from Sigma (U.K.), Hank's balanced salt solution (HBSS) from Flow Laboratories (U.K.), Lymphoprep from Nycomed Pharma SA (Norway), RPMI-1640 from Gibco BRL, guanidinium isothiocyanate from Fluka Chemicals Ltd (U.K.), Taq DNA polymerase–AmpliTaq from Perkin Elmer Cetus (U.S.A.), Perfect Match from Stratagene Ltd (Cambridge, U.K.) and bovine-serum albumin (BSA, RNase- and DNase-free) from Anglian Biotec Ltd (U.K.). Avian myeloblastosis virus (AMV) reverse transcriptase, oligo-dT, dNTP and RNA guard were from Pharmacia LKB Biotechnology (Sweden).

Purification of PBMC

Fifty millilitres of venous blood from nine atopic (six males, three females; age range 22–55 years) and seven non-atopic subjects (four males, three females; age range 24–41 years) was collected in 40 units of heparin per ml, mixed with an equal volume of HBSS, overlaid on one-third vol. of Lymphoprep and sedimented at 400 ***g*** for 20 min. Mononuclear cells were collected from the interphase and washed three times with two

vols of HBSS per original volume of blood. Cells were then suspended at 2×10^6 cells per ml in RPMI-1640 containing 2 mM L-glutamine and 10% foetal calf serum (FCS) in 50-ml Falcon tubes, 5–10 ml per tube, and incubated with or without activation with PMA at 37°, in 5% CO_2, with the tubes tilted to the near-horizontal position. Atopy was defined by the presence of at least two positive reactions (wheal 3 mm greater than in saline controls) to a skin-prick test with the following allergens: cat fur, dog hair, grass pollen and *Dermatophagoides pteronyssinus*. Eosinophilia was assessed by counting eosinophils in a haemocytometer after Kimura staining, and expressing them as a percentage of the total white blood cell population. All subjects gave informed consent, and the study was approved by the Guy's Hospital Ethical Committee.

Total cellular RNA

Total cellular RNA was isolated by the guanidinium isothiocyanate method,[11] precipitated twice with 1/10 vol. of 3 M sodium acetate pH 5·2 and 2·5 vols of ethanol and dissolved in 25 mM 2-mercaptoethanol containing 0·5 U/μl RNA guard. The yields were usually 2–2·5 μg of RNA per 10^6 cells.

Reverse transcription (RT) and polymerase chain reaction (PCR)

RT and PCR were performed according to the protocol for PCR recommended by Cetus, with modifications.

RT. Thirty microlitres of 'RT mix' per sample was prepared as 4 μl of 10 × RT buffer (500 mM Tris-HCl pH 8·0, 500 mM KCl, 50 mM $MgCl_2$), 5 μl of 1 μg/μl oligo-dT, 1 μl of 200 mM dithiothreitol (DTT) 1·8 μl of dNTP (25 mM each), 2 μl of 1 μg/μl BSA, 0·5 μl of 40 U/μl RNA guard, 1·2 μl of 18 U/ml AMV reverse transcriptase and 14·5 μl of water. RT mix was made for all samples, vortexed briefly and aliquoted as 30 μl per tube plus 10 μl of 2·5 μg/μl total RNA. Tubes were then vortexed again, spun briefly in a microcentrifuge and left for 10 min at room temperature and then for 30 min at 42°. Reverse transcriptase was inactivated at 72° for 10 min and samples were used for PCR or were kept frozen at −60° for several weeks. Aliquots of 10 μl were run on an agarose gel to ensure that RNA had not been degraded during the reverse transcription.

PCR. Each reaction consisted of 5 μl of the reverse-transcribed RNA (0·5 μg of total RNA) and 35 μl of PCR mix (25·6 μl of water + 3·5 μl of 10 × RT buffer + 2·5 μl 20 μM up-primer + 2·5 μl of 20 μM down-primer + 0·5 μl of 1 U/μl Perfect Match + 0·4 μl of 5 U/μl AmpliTaq). Alternatively, two or three pairs of primers were added to one reaction tube, since there was no cross-hybridization between them. However, the β-actin primers were added as 2·5 μl of 2 μM instead of 20 μM each. PCR mix was prepared for all reactions, vortexed and aliquoted (35 μl per tube) on ice. Five microlitres of reverse-transcribed RNA samples was added to each tube, vortexed, spun briefly and overlaid with mineral oil. The tubes were then transferred to the thermal cycler preheated to 72°. After the last sample was transferred to the cycler, PCR was started with a 94° step for 2 min, followed by up to 26 PCR cycles (1 min at 94°, 2 min at 60° and 2 min at 72°).

The primers used in this study were: (i) 5′-GAGCCATGAGGATGCTTCTGC-3′ and 5-′GGAATCCTCAGAGT'CTCATTGG-3′, which spanned a 163-nucleotide (nt) fragment of the first and second exons of the IL-5 gene; (ii) 5′CTAAAGTTCTCTGGAGGATGTGG-3′ and 5′TTCTACTGTTTCATT'CATCTCAGC-3′, which covered a 191-nt fragment of the first and second exons of the GM-CSF gene; and (iii) 5′-CGCGAGAAGATGACCCAG'ATC-3′ and 5′-ATCACGATGCCAGTGGTACGG-3′, which covered a 113-nt fragment of the third and fourth exons of the β-actin gene. One primer of each pair spanned across a splicing junction (indicated by ') to prevent priming of genomic DNA. Thus the IL-5 and GM-CSF pairs could only amplify genomic DNA templates at annealing temperatures below 35°, and the β-actin pair could not amplify genomic DNA.

Aliquots (3–8 μl each) were taken from each sample after 17, 22 and 24 or 25 cycles and were run on 3·2% agarose gel in glycine buffer, stained with ethidium bromide. Photographs were taken at 280 nm illumination on Agfa APX25/15° roll film The negatives were traced on a Molecular Dynamics Image Quant densitometer. The quantification of the bands was realized by interpolation of the area of the optical densities of the bands from the marker bands (pUC18/H*pa*II). Molecular weight vs. band area plots of the marker fragments were also used for assessment of the region of linear response. IL-5,[18] β-actin[19] and GM-CSF[20] cDNA plasmids were used as controls.

RESULTS

PCR amplification of cDNA

A series of PCR amplifications were performed on 10^4, 10^5 and 10^6 copies of IL-5, GM-CSF and β-actin cDNA in 40 μl of reaction solution for 20–29 cycles. The amplification per cycle was calculated for each. Figure 1a shows an agarose gel of aliquots of the IL-5 samples after 20, 23, 26 and 29 cycles. We did not detect any decrease in the amplification caused by exhaustion of the enzyme or hydrolysis of the dNTP up to 26 cycles. However, we found that the amplification of each fragment became saturated after a certain number of cycles. Thus the sample with 10^6 copies became saturated after 20 cycles, while 10^5 and 10^4 copy samples were saturated after 23 and 27 cycles, respectively. Similar results were obtained wih the other two cDNA. Thus saturation depended only on the final concentration of the fragment, and it could not be avoided by the addition of more enzyme or dNTP. The amplification improved slightly after dilution with buffer. In Fig. 1b are plotted the calculated amplifications per cycle vs. yield. It is seen that the saturation started at about 0·05 μM amplified fragment (about 200 ng).

IL-5 and GM-CSF mRNA levels in activated PBMC

The levels of IL-5 and GM-CSF mRNA in PBMC were monitored by PCR amplification of reverse-transcribed RNA. Aliquots of 3–8 μl were taken after 22 and 24 PCR cycles and were then run on a 3·2% agarose gel. mRNA for both genes from freshly isolated PBMC from atopic and non-atopic individuals was less than 500 copies per μg of total RNA. There was no increase in mRNA when cells were kept as 5-ml aliquots at 10^6 cells/ml for 24 hr in RPMI-1640, 2 mM L-glutamine, 10% FCS, at 37° in 50-ml Falcon tubes. Activation of the cells with PMA (5 ng/ml) for 20 hr increased the level of IL-5 and GM-CSF mRNA by several orders of magnitude, and there was a marked difference between the levels of mRNA for both genes in some atopic patients and those in non-atopic control subjects (Fig. 2). There was a significant correlation between the levels of

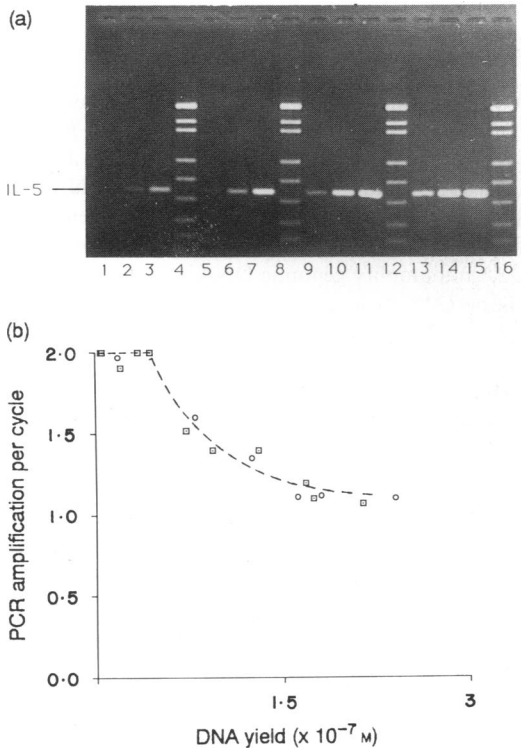

Figure 1. (a) Agarose gel of 10^4, 10^5 and 10^6 copies of IL-5 cDNA that was PCR amplified for 20 cycles (lanes 1, 2 and 3); for 23 cycles (lanes 5, 6 and 7); for 26 cycles (lanes 9, 10 and 11); and for 29 cycles (lanes 13, 14 and 15). Lanes 4, 8, 12 and 16 are DNA marker fragments (pUC18/*Hpa*II). (b) Calculated amplification per PCR cycle vs. yield in molar concentration. (□) From the gel in (a); (○) from a separate experiment.

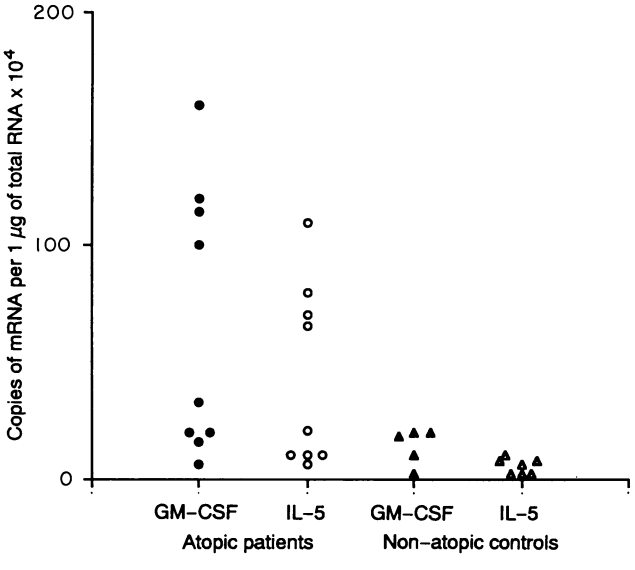

Figure 2. IL-5 and GM-CSF mRNA (copies per μg of total RNA) from PBMC of atopic and non-atopic subjects after activation with 5 ng/ml PMA for 20 hr (two of the non-atopic subjects were monitored only for IL-5 mRNA).

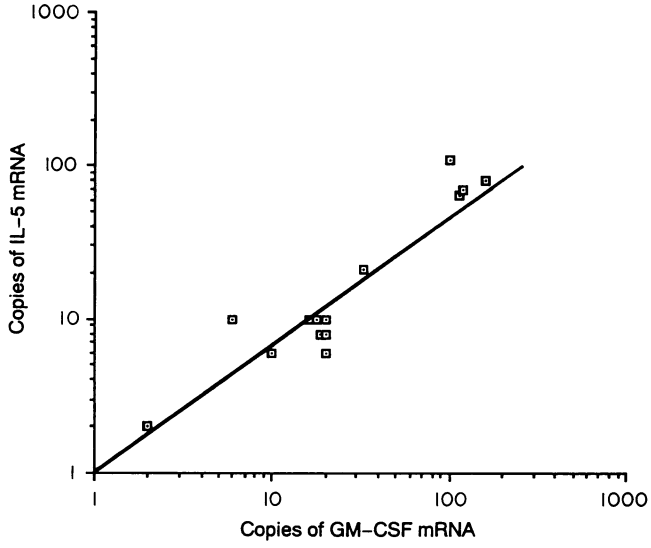

Figure 3. Copies of IL-5 mRNA vs. copies of GM-CSF mRNA from PBMC of all the individuals studied.

IL-5 and GM-CSF mRNA in activated cells (Fig. 3). Thus all high IL-5 producers were also high GM-CSF producers. Addition of cycloheximide prior to PMA activation completely abolished the accumulation of both mRNA in PBMC, suggesting that the activation of IL-5 expression requires expression of another gene(s).

Correlation of IL-5 and GM-CSF mRNA levels with peripheral blood eosinophilia

There was no linear relationship between the level of IL-5 and GM-CSF mRNA and the percentage blood eosinophilia ($r=0{\cdot}47$, $P>0{\cdot}05$, and $r=0{\cdot}24$, $P>0{\cdot}05$, respectively). All subjects who produced less than 1×10^5 copies of IL-5 mRNA per μg of RNA after PMA activation had less than 1% eosinophilia. However, one of the high IL-5 producers (7×10^5 copies mRNA per μg of RNA) had only 2·5% eosinophilia, and a moderate IL-5 producer (2×10^5 copiers per μg of RNA) had 16% eosinophilia.

Kinetics of IL-5 and GM-CSF mRNA levels after activation with PMA

In Fig. 4a and b are shown agarose gels of the time-course of increase in IL-5 and GM-CSF mRNA levels, after activation with PMA, for a subject who produced high levels of IL-5 and GM-CSF mRNA. PCR amplification was carried out with three pairs of primers: for IL-5, for GM-CSF (1·25 μM each) and for β-actin (0·125 μM). Only the β-actin fragments were seen after 17 cycles (Fig. 4a). After 22 cycles, IL-5 and GM-CSF fragments appeared and β-actin fragments were saturated (Fig. 4b). The integrated optical densities of the bands obtained from scanning of the photographic negatives were used to calculated the numbers of IL-5 and GM-CSF mRNA copies (Fig. 4c). A very similar time-course was found for all individuals studied, independent of whether they were high or low IL-5 producers. Figure 4d shows time-courses of high and low IL-5 producers presented as percentages of the maximum level of mRNA.

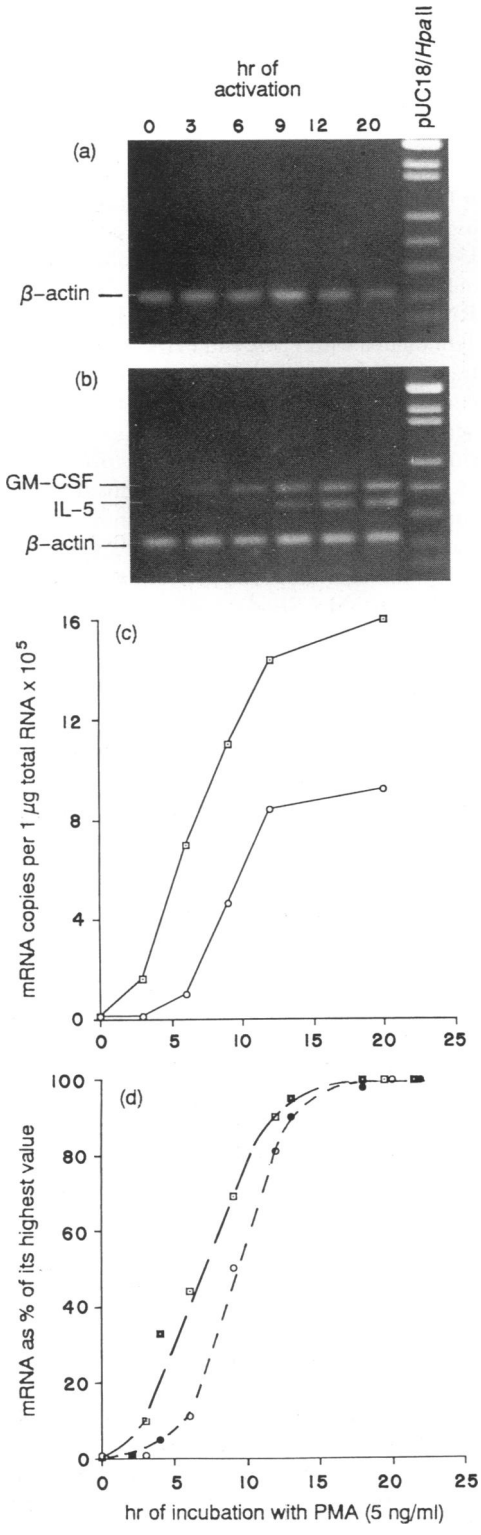

Figure 4. (a, b) Agarose gels of PCR-amplified mRNA of a high IL-5 and GM-CSF producer after activation with 5 ng/ml PMA for 0, 3, 6, 12 and 20 hr. PCR was carried out with primers for the three mRNA simultaneously: (a) after 17 cycles; (b) after 22 cycles. (c) Time-course of IL-5 and GM-CSF mRNA (copies per μg of total RNA) calculated from (b). ⊡, GM-CSF; ○, IL-5. (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. ⊡, GM-CSF high; ○, IL-5 high; □, GM-CSF low; ●, IL-5 low.

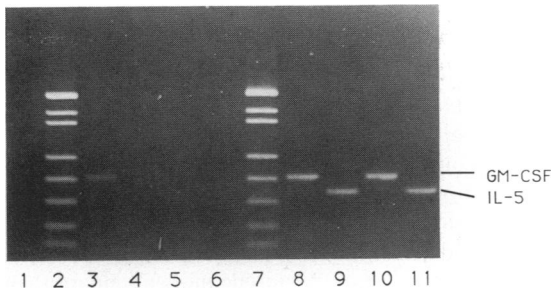

Figure 5. Agarose gels of PCR-amplified cDNA samples of cells that were incubated with: 1, control; 3 and 4, rhGM-CSF (1 ng/ml) and PMA (5 ng/ml) for 2 hr; 5 and 6, rhGM-CSF (1 ng/ml) for 20 hr; 8 and 9, anti-GM-CSF (1/1000 dilution) and PMA (5 ng/ml) for 20 hr; 10 and 11, PMA (5 ng/ml) for 20 hr; 2 and 7, DNA marker (pUC18/*Hpa*II); 1, 4, 6, 9 and 11, amplified with IL-5 primers; 3, 5, 8 and 10, amplified with GM-CSF primers.

Transcription of IL-5 and GM-CSF started about 3 hr after activation and continued to increase until 15 hr, after which time it remained stable for another 24 hr.

There was a small but significant lag period of 3 hr 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. In order to test the latter possibility we incubated mononuclear cells in presence of (i) 1 ng/ml rhGM-CSF for 2 and 20 hr, (ii) 1 ng/ml recombinant human (rh)GM-CSF and 5 ng/ml PMA for 2 and 20 hr, (iii) anti-GM-CSF monoclonal antibody (1:1000 dilution) and 5 ng/ml PMA for 20 hr and (iv) 5 ng/ml PMA alone. Figure 5 shows an agarose gel of PCR-amplified cDNA of these cells. It is seen that rhGM-CSF alone did not result in the appearance of IL-5 mRNA for up to 20 hr. After 2 hr of 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 mAb did not inhibit PMA activation of either gene.

The same kinetics was observed when the cells were activated with 5 or 10 ng/ml PMA, or with PMA + 1 μg/ml ionomycin. Higher concentrations of PMA did not shift the time-course towards shorter times but reduced the viability of the cells after 24 hr with less consistent results.

In another experiment, cells were washed 3 hr after activation and then resuspended in fresh medium without PMA. The level of both mRNA increased in the same way despite removal of PMA. After 20 hr the mRNA were close to those seen in the control sample in which PMA was not removed from the incubation mixture (results not shown).

DISCUSSION

The level of mRNA was studied by PCR. Since we have made a number of modifications to the published protocol for PCR, which led to very reproducible quantitative results, we will discuss them in some detail:

(i) Total RNA was reverse transcribed with oligo-dT primer since we found that, when reverse transcription was primed with specific primers, PCR amplification gave a number of 'ghost bands'. Priming with oligo-dT led to a much cleaner picture. It is likely that a 42° (required by the reverse transcriptase) the

specific oligonucleotides primed some mismatched or shorter sequences.

(ii) PCR could be carried out for two or three different mRNA simultaneously in the same tube if they were present in similar numbers. The yield of IL-5 and GM-CSF fragments generated by our primers did not differ, irrespective of whether the amplification was carried out in the same tube or in separate tubes. However, the presence of β-actin primers significantly inhibited the amplification of these transcripts. Since β-actin mRNA was two orders of magnitude more abundant than the two cytokine mRNA, this inhibition was probably caused by competition for the enzyme. This problem was avoided when the β-actin primers were used at a concentration of 0·125 μM. Aliquots were taken at an early stage (after 16 or 17 cycles), in order to monitor the yield of the β-actin fragment before it became saturated.

(iii) In this study we have used a maximum of 26 PCR cycles, which resulted in detection of one molecule of mRNA per 25 cells. In our experience, if larger number of cycles were required for detection of mRNA, either the reverse transcription or the PCR was inefficient and the reproducibility of the results was poor.

(iv) Saturation of the amplification started when the yield of the fragments reached 0·05 μM. It seems most likely that self-annealing of the whole fragments was more favourable than annealing with the oligonucleotide primers. Thus when the concentration of the amplified fragments reached a sufficient level the primers were inhibited. Indeed, for 0·05 μM fragments ($=1{\cdot}6\times10^{-5}$ M nucleotides), a 2-min annealing time gave $Cot=2\times10^{-3}$, which is sufficient for the renaturation of fragments a few hundred nucleotides long. Errors resulting from saturation of amplification were avoided by running aliquots after two consecutive cycles (e.g. 22 and 23 cycles) and then comparing the intensities of the bands.

The basal expression of IL-5 and GM-CSF mRNA in freshly purified PBMC from atopic as well as non-atopic individuals was less than 500 copies per μg of total RNA or less than one copy per 1000 cells. Activation with PMA alone or PMA and ionomycin together increased the mRNA levels by several orders of magnitude. There was a considerable difference between the levels of both mRNA in some atopic and non-atopic individuals. However, there was no linear relationship between the level of mRNA of each of the two cytokines and the peripheral blood eosinophilia. It is probable that levels of the secreted protein will be more closely related to circulating eosinophil counts. In addition, the expression of cytokine receptors on eosinophils may dictate the response of these cells to the secreted molecules. A similar enhanced secretion of IL-5 but not of GM-CSF has been found in patients with helminth-induced eosinophilia.[10] Our results show that the same individuals who have a high expression of IL-5 mRNA also have high GM-CSF mRNA expression. However, Limaye *et al.*[10] monitored secreted proteins, whereas we have monitored mRNA. Since it is very unlikely that there is an attenuation of GM-CSF expression at translational level in helminth-infested patients only, this difference between the two studies may reflect a different mechanism of up-regulation of these cytokines in atopic and in helminth-infested subjects.

We have found that activation of IL-5 expression in T cells requires protein synthesis, as does GM-CSF,[12] and it is not surprising that its expression started relatively late. It is interesting, however, that the levels of both IL-5 and GM-CSF mRNA increased for another 15 hr even after PMA had been washed out. This suggests that PMA was only needed during the first few hours to activate another gene(s) and that this controlled the long-lasting transcription of IL-5 and GM-CSF genes in PBMC. Similar long-lasting expressions of IL-5 have been observed in murine T-helper type 2 clones activated with IL-2, IL-4 or anti-CD3,[8] or in human PBMC stimulated with PMA and ionomycin.[10] A similar long-lasting accumulation of IL-2 mRNA has been observed in human PBMC[13] and purified T cells[14] stimulated with PMA and PHA. The present results are in contrast to the monitored expression of GM-CSF in a number of other cells. In mouse peritoneal macrophages stimulated with lipopolysaccharide, phagocytosis and adherence in the presence of fibronectin, *in vitro* transcription of GM-CSF starts between 1 and 2 hr after activation, and reaches a maximum between 2 and 8 hr.[12] After stimulation of a murine mastocyte line with PMA and/or ionophore, GM-CSF accumulates during the first hour of activation.[15] A similar rapid accumulation of GM-CSF mRNA was observed when bone marrow mast cells were activated through the high-affinity receptor for IgE. The maximum mRNA level was observed after 30 min, and it was no longer detectable after 2 hr.[16] Thus the cumulative data suggest that the time-course of IL-5 and GM-CSF activation is tissue specific.

There was a 3-hr delay in IL-5 gene activation as compared with that of GM-CSF. However, the possibility that expression of GM-CSF was required for the increase in the IL-5 mRNA level is unlikely, since rhGM-CSF alone did not cause IL-5 activation, rhGM-CSF and PMA did not shift the IL-5 kinetics toward shorter times, and the presence of anti-GM-CSF mAb in the media did not prevent IL-5 mRNA activation by PMA. The concentrations of rhGM-CSF and anti-GM-CSF mAb were selected as sufficient for rhGM-CSF to support eosinophil for 1 week and for anti-GM-CSF mAb to neutralize the effect of GM-CSF.[17] The different kinetics of activation of GM-CSF and IL-5 suggest that there may be an additional signal(s) in the regulation of the transciption of one of them, or they may be expressed by different sets of cells.

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