Immunology 1995 **84** 410–415

IL-4 enhances IL-3 and IL-8 gene expression in a human leukemic mast cell line

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SUMMARY

We examined the capacity of interleukin (IL)-4 to induce or enhance the expression of certain cytokines in resting and activated cells of the HMC-1 human leukemic mast cell line. The HMC-1 mast cells were cultured with or without recombinant human IL-4 and then activated with the calcium ionophore ionomycin. Stimulation of non-IL-4-treated cells with ionomycin (10 μM) for periods of 30 min to 8 hr induced expression of mRNA encoding IL-3, IL-4 and IL-8 but was without effect on levels of mRNA for tumour necrosis factor (TNF)-α or β-actin. Culture of the cells with IL-4 (100 ng/ml) for 24 hr led to a small increase in resting levels of mRNA for IL-3 and IL-8 but not for IL-4, TNF-α or β-actin. More notably, the IL-4 treatment produced a pronounced elevation of mRNA for IL-3 and IL-8 when the cells were subsequently activated with ionomycin. The IL-4 treatment produced a negligible effect on IL-4 mRNA, and no effect on TNF-α or β-actin mRNA levels in ionomycin-activated cells. Quantitation of cDNA by competitive polymerase chain reaction (PCR) revealed that the IL-4 treatment produced a sixfold increase in ionomycin-induced levels of cellular IL-3 mRNA, a fourfold increase in induced IL-8 mRNA and less than a twofold increase in induced IL-4 mRNA. The IL-4 treatment led to a 15- to 20-fold increase in ionomycin-induced secretion of IL-3 product and a doubling of induced IL-8 product. These effects of IL-4 were not associated with increased mast cell numbers. We conclude that IL-4 alone is a weak activator of IL-3 and IL-8 gene expression in mast cells, but is able to enhance activation signals in stimulated mast cells leading to transcription and secretion of these two cytokines.

INTRODUCTION

Activated mast cells of mouse origin express mRNA, and in some cases protein product, for multiple cytokines including interleukin (IL)-1α, IL-3, IL-4, IL-5, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and tumour tecrosis factor-α (TNF-α), as well as some members of the chemokine family such as macrophage inflammatory proteins (MIP)1α and 1β.[1–5] Information is emerging with regard to cytokine expression in mast cells of human origin, although the picture is less complete. For example, human long-term cultured mast/basophil-like cells[6] and human-skin mast cells[7,8] express TNF-α mRNA and protein product; human-lung mast cells express and release IL-4 protein;[9] the human mast cell line HMC-1 expresses mRNA and protein for IL-1β, TNF-α[10] and multiple chemokines including IL-8,[11,12] MIP-1β and RANTES.[12]

IL-4 is a T cell- and mast cell-derived cytokine[4,9,13,14] with wide-ranging immune regulatory effects; most notably, it acts as a B-cell growth and differentiation factor[14] and in T cells promotes production of selected cytokines such as IL-4, IL-5, GM-CSF and IL-10 that together define the Th2 subset.[14] IL-4 also exerts effects on tissue mast cells *in vitro*: it acts as a co-factor (with IL-3) for their growth,[15] and alone enhances their exocytotic secretory responsiveness to IgE-mediated stimulation.[16] Considering the above properties of IL-4, we tested the hypothesis that IL-4 might influence cytokine gene expression in mast cells. Our results show that IL-4 enhances induced gene expression for IL-3 and IL-8 in a human mast cell line.

Received 15 September 1994; revised 19 October 1994; accepted 27 October 1994.

Abbreviations: FCS, fetal calf serum; HMC-1, a human mast cell leukemia cell line; IFN-γ, interferon-γ; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; PCR, polymerase chain reaction; RT, reverse transcription; TNF-α, tumour tecrosis factor-α.

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MATERIALS AND METHODS

Cells

Cells of the growth factor-independent human leukemia-derived mast cell line HMC-1[17] were cultured in complete Iscove's modified Dulbecco's medium (IMDM) containing

10% heat-inactivated fetal calf serum (FCS), 1·2 mM monothioglycerol, 100 μg/ml penicillin and 100 U/ml streptomycin. The cells were routinely maintained at a density of 10^6/ml at 37° in 5% CO_2.

Cell treatment, challenge and RNA extraction

HMC-1 cells ($1-2 \times 10^6$/ml) were cultured with or without recombinant human IL-4 (100 ng/ml, R&D Systems, Minneapolis, MN; contains < 0·1 ng endotoxin per μg). After 24 hr the cells were sedimented (150 ***g***, 5 min) and resuspended at 2 to 4×10^6 cells/ml in complete IMDM at 37°. The cells were challenged for different time periods with 10 μM ionomycin or control complete IMDM. The cells were sedimented in microfuge tubes (2000 ***g***, 5 min) and the cell pellet lysed with 200 μl of ice cold solution A containing 10 mM Tris-HCl, 0·15 M NaCl, 1·5 mM $MgCl_2$, 0·65% NP-40 and 0·2 U/ml RNasin (recombinant RNase inhibitor, Promega, Southampton, UK), pH 7·5. The cell nuclei were pelleted (6500 rpm, 5 min) and the supernatant fraction mixed with 200 μl of solution B containing 7 M urea, 1% SDS, 0·35 M NaCl, 10 mM EDTA and 10 mM Tris-HCl, pH 7·5. To separate cellular RNA and contaminating DNA the extracts were mixed with 400 μl phenol/chloroform/isoamyl alcohol (25/24/1) and spun in a microcentrifuge at 6000 ***g*** for 10 min. The aqueous layer containing the RNA was separated and RNA precipitated overnight at −20° with 400 μl ethanol and 40 μl of 3 M sodium acetate. The RNA was pelleted (6000 ***g***, 15 min), air dried, then dissolved in 20 μl water at 60° for 5 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

Five-microlitre aliquots of the RNA solution were reverse transcribed using a deoxythymidine primer of 20 nucleotides and 15 U reverse transcriptase (Promega). Selected sequences in 5 μl aliquots of cDNA were amplified by PCR over 30 cycles using primers for β-actin, IL-3, IL-4, IL-8 and TNF-α. The reactants were cycled at 92° for 1 min, 50° for 1 min and 72° for 2 min. The sequences of the primers were: β-actin, 5′-CTGGCACCCAGCACAATGAAG-3′ and 5′-ACCGACTGCTGTCACCTTCA-3′, amplified fragment 362 bp; IL-3, 5′-CTGCTCTAACATGATCGATG-3′ and 5′-GAGAACGAGCTGGACGTTGG-3′, amplified fragment 378 bp; IL-4, 5′-CTGCAAATCGACACCTATTA-3′ and 5′-GATCGTCTTTAGCCTTTC-3′, amplified fragment 449 bp; IL-8, 5′-GCAGCTCTGTGTGAAGGTGCA-3′ and 5′-GAATTCTCAGCCCTCTTCAA-3′, amplified fragment 248 bp; TNF-α, 5′-CGAGTGACAAGCCTGTAGCC-3′ and 5′-CTACCAGACCAAGGTCAAC-3′, amplified fragment 254 bp. PCR reaction products were separated at 50 V for 2 hr on a 2% agarose gel in Tris-acetate-ethylenediaminetetracetic acid (EDTA) buffer containing 0·5 μg/ml ethidium bromide. DNA molecular weight markers (1 μg, Promega) were run in parallel. The gels were visualized and photographed under UV light. The identities of the PCR products, all of which migrated according to their predicted size, were confirmed by restriction enzyme digestion giving predicted fragment sizes (IL-3 PCR product after *Eco*RI, 378 bp to 107 bp + 271 bp, after MboII to 95 bp + 283 bp; IL-4 after EcoRI, 449 bp to 70 bp + 379 bp, after MboII to 47 bp + 117 bp + 285 bp; IL-8 after *Hin*dIII, 248 bp to 91 bp + 157 bp; TNF-α after MboII, 254 bp to 96 bp + 158 bp). The quantity of cDNA added to PCR reactions was adjusted such that the amount of product was linearly related to the amount of starting cDNA for each of the PCR products over the number of cycles used.

Quantitative PCR

cDNA levels were quantified by a competitive PCR method.[18] PCR 'mimics' were made using a mimic construction kit (Clontech, Cambridge, MA). Cytokine mimics were prepared for IL-3, IL-4 and IL-8 by amplification of a fragment of *v-erbB* gene DNA in a PCR reaction over 16–21 cycles with composite primers complimentary to both the *v-erbB* gene DNA and the relevant cytokine sequence. For this preparation, the reactants were cycled at 94° for 45 seconds, 60° for 45 seconds and 72° for 90 seconds. The sequences of the composite primers were: IL-3, 5′-CTGCTCTAACATGATCGATGGGAGAAGGGAGAGCGTTTGC-3′ and 5′-GAGAACGAGCTGGACGTTGGGGAGCTTTGAGATTGCTGTG-3′; IL-4, 5′-CTGCAAATCGACACCTATTAGCTACAAACTGCATTGACAG-3′ and 5′-GATCGTCTTTAGCCTTTCGGAGCTTTGAGATTGCTGTG-3′; IL-8, 5′-GCAGCTCTGTGTGAAGGTGCAGGAGAAGGGAGAGCGTTTGC-3′ and 5′-GAATTCTCAGCCCTCTTCAAGGAGCTTTGAGATTGCTGTG-3′. Mimics produced during the first round of PCR were diluted and used in a second round of PCR for 18 cycles with cytokine-specific primers to ensure that all the mimic molecules had cytokine primer binding sites. The mimics were separated from the primers on gel-filtration matrices (Clontech) and quantified by comparisons of electrophoretic band intensities with those of known quantities of DNA molecular weight markers. Known quantities of mimic (twofold dilutions) were spiked in PCR reactions together with target cDNA. Gel photographs were scanned by laser densitometry (LKB Ultroscan XL, Pharmacia, Uppsala, Sweden) and the area under the curve of band peaks calculated (GelScan XL 2·1 software, Pharmacia, Uppsala, Sweden) and plotted. The point of intersection of the plots for mimic and target DNA was taken as a measure of the relative amount of starting cDNA. We obtained a close relationship between the amount of starting mimic cDNA and the amount of PCR product measured densitometrically. Although DNA of different lengths may be amplified with different efficiency, the relative amounts of starting target cDNA could be measured reliably judging by curve linearity and consistent results between experiments.

Cell proliferation

HMC-1 cells were cultured at 5×10^5/ml in quintuplicate 111 μl volumes in 96-well tissue culture plates with a range of concentrations of IL-4 up to 100 ng/ml for 24 hr. Proliferation was measured as cellular incorporation of [6-^{3}H]thymidine (Amersham Life Sciences, Amersham UK, sp act 26 Ci/mM) added to the cells (5 μCi/ml) 4 hr before the end of the culture period.

Assays for cytokine release

HMC-1 cells were cultured at a density of 4×10^6/ml in complete IMDM, with or without 100 ng/ml IL-4 for 24 hr. The cells were sedimented and resuspended in fresh medium at 37° for challenge with 10 μM ionomycin or medium alone for 1 hr, then pelleted, resuspended to the same density in complete IMDM and returned to culture at 37°. Aliquots of cell suspension were removed at 2, 4, 6, 8 and 24 hr after challenge, centrifuged (200 ***g***, 5 min) and the cell-free supernatant fractions assayed for IL-3 and IL-8 protein by enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

RESULTS

Expression of mRNA for IL-3, IL-4, IL-8 and TNF-α in unstimulated and activated mast cells

Messenger RNA encoding the cytokines IL-3, IL-4, IL-8 and TNF-α was detected by reverse transcriptase (RT)-PCR in cytoplasmic lysates from unstimulated HMC-1 cells although the intensity of the PCR product bands (particularly IL-8) was often weak after 30 PCR cycles (Fig. 1). Stimulation of the cells with ionomycin (10 μM) induced expression of mRNA for IL-3 (13/14 experiments), IL-8 (8/8) and IL-4 (7/10), but rarely TNF-α (2/14). In all experiments, levels of expression of mRNA for the 'housekeeping' gene β-actin were not influenced by ionomycin. (See Fig. 1 for results of an experiment in which cells were stimulated with ionomycin for 4 hr.) Following ionomycin activation of the cells, levels of mRNA for IL-3 (Fig. 2) and IL-8 increased steadily for 4 to 8 hr, while levels of IL-4 mRNA peaked after 1 to 4 hr.

IL-4 enhances transcription of IL-3 and IL-8

Culture of HMC-1 cells with IL-4 (100 ng/ml) for 24 hr led to a small but detectable increase in mRNA for IL-3 and IL-8 but not IL-4, TNF-α or β-actin in unstimulated cells, and produced a marked enhancement of mRNA expression for IL-3 and IL-8 when the cells were subsequently stimulated by ionomycin (Figs 1–3 for IL-3, 1 and 3 for IL-8). IL-4 produced a smaller effect on induced expression of IL-4 mRNA, and was totally without effect on induced expression of mRNA for TNF-α and β-actin (Fig. 1). Similar results to those shown in Fig. 1 were seen in at least six independent experiments. The enhancing effect of IL-4 is illustrated further in Fig. 2 which shows the time course of ionomycin-induced expression of IL-3 mRNA in IL-4-treated and control cells. It can be seen that IL-4 enhanced the expression of IL-3 mRNA in resting cells but more markedly in cells activated with ionomycin for various times between 30 min and 4 hr, while levels of β-actin remained constant under all conditions.

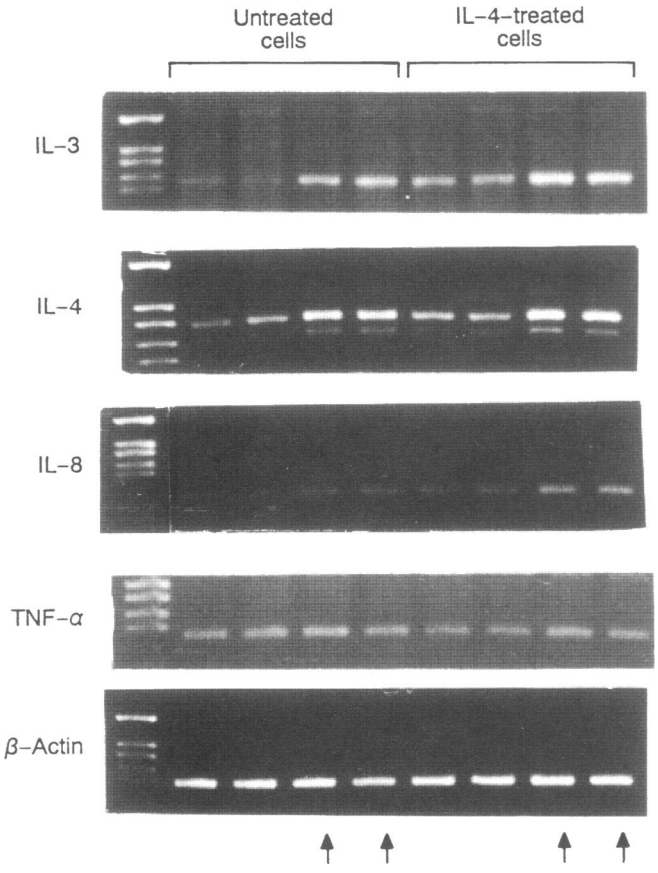

Figure 1. RT-PCR analysis of mRNA for IL-3, IL-4, IL-8, TNF-α and β-actin in HMC-1 mast cells. Cells were cultured with or without IL-4 (100 ng/ml, 24 hr) and then challenged with ionomycin (10 μM) or control medium for 4 hr. Arrows indicate ionomycin stimulation (lanes 4, 5, 8, 9). Results of duplicate cell cultures from a representative experiment are shown. DNA molecular weight markers were run in the left hand lane.

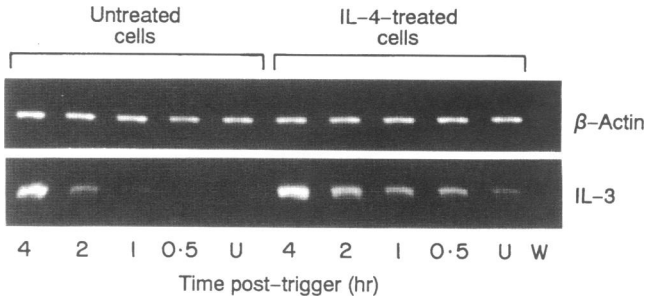

Figure 2. RT-PCR kinetic analysis of mRNA for IL-3 and β-actin from activated HMC-1 mast cells. The cells were pretreated with or without IL-4 (100 ng/ml, 24 hr) and then stimulated with ionomycin. RNA was extracted from the cells at different time points post challenge. U = unstimulated cells; W = water control; times are post addition of ionomycin to the cells.

Quantitation of the effects of ionomycin and/or IL-4 on levels of mRNA for IL-3, IL-4 and IL-8

Competitive PCR and laser densitometry were used to quantify the extent of amplification of mRNA seen after ionomycin challenge (4 hr) and/or IL-4 pretreatment (24 hr) of the cells. In the representative experiment shown in Fig. 3, mRNA for IL-3 was increased 17-fold, IL-8 by at least fourfold, while that for IL-4 was doubled after ionomycin challenge. Treatment of the cells with IL-4 (100 ng/ml, 24 hr) enhanced induced expression of IL-3 mRNA by sixfold (Fig. 3a), IL-8 mRNA by fourfold (Fig. 3c) while IL-4 mRNA was less than doubled (Fig. 3b).

Effects of IL-4 on IL-3 and IL-8 secretion

IL-3 and IL-8 were detected in the supernatant fractions of HMC-1 cells stimulated with ionomycin for 1 hr, then washed and cultured for various times (Fig. 4). Peak IL-3 release from non-IL-4-treated cells was reached 6 hr after challenge and remained steady at 350–400 pg/ml thereafter for the 24 hr duration of the experiment (Fig. 4a). Pretreatment of the cells with IL-4 (100 ng/ml, 24 hr) led to a dramatic increase in ionomycin-induced release of IL-3 (Fig. 4a). Induced release of IL-3 from the IL-4-treated cells continued to rise during the 24 hr period of the experiment to reach levels of 4–6 ng/ml, representing a 15- to 20-fold increase over levels released from

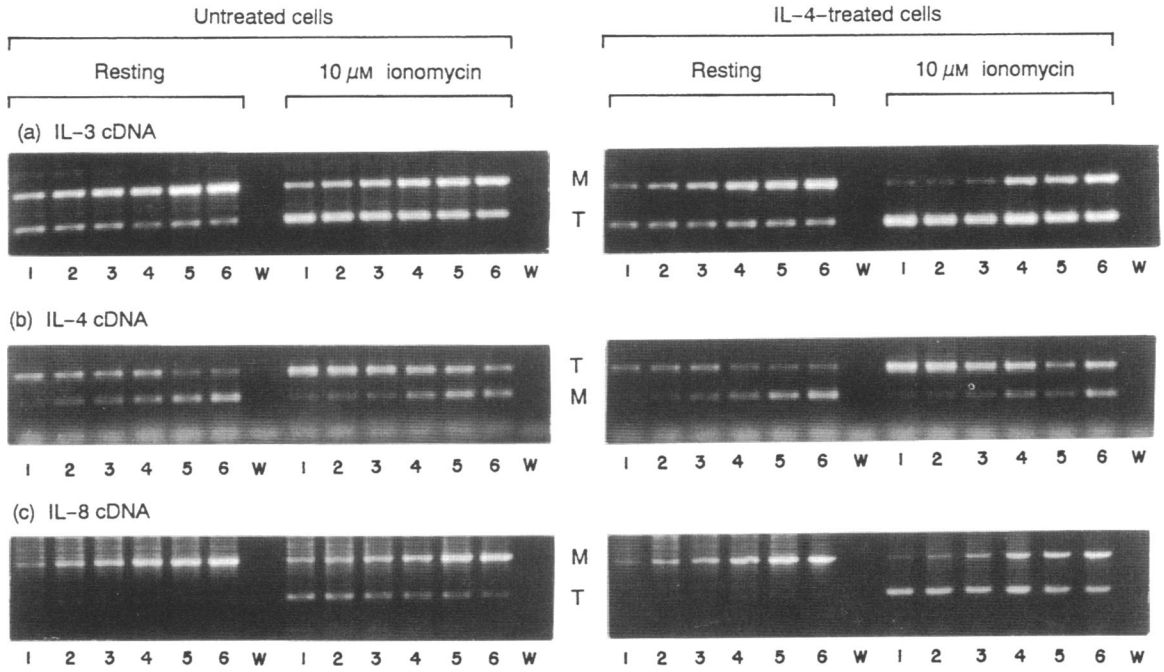

Figure 3. Quantitation by RT-competitive PCR of ionomycin- and IL-4-induced changes in expression of cytokine mRNA. HMC-1 cells were cultured with or without IL-4 for 24 hr, then washed and challenged with ionomycin or control medium for 4 hr. (a) IL-3 cDNA, (b) IL-4 cDNA and (c) IL-8 cDNA. M = mimic cDNA; T = target cDNA; W = water control. Lanes 1–6 show progressive doubling of the amount of mimic DNA spiked into PCR tubes containing a constant amount of target cDNA.

non-IL-4-treated cells (Fig. 4a). IL-4-treated cells stimulated with ionomycin for 24 hr released 1–1·5 fg of IL-3 per cell. Cells that were not stimulated with ionomycin failed to release detectable IL-3 regardless of whether or not they were pretreated with IL-4 (Fig. 4a). Ionomycin-activated cells released IL-8 with peak levels reached at 6 hr after stimulation. By 24 hr supernatant levels had fallen quite markedly, indicating instability of IL-8 in the cell culture. Pretreatment of the cells with IL-4 (100 ng/ml, 24 hr) doubled levels of ionomycin-induced IL-8 release (Fig. 4b). Peak levels of released IL-8 from IL-4-treated cells reached 700 pg/ml, representing 0·17 fg IL-8 released per cell. Unstimulated cells failed to release detectable IL-8 regardless of whether they were pretreated with IL-4 (Fig. 4b).

The relatively slow release of IL-3 and IL-8 (peaking at 24 or 6 hr respectively after cell stimulation) is suggestive of *de novo* protein synthesis, and this would be consistent with the time course of induction of mRNA for these cytokines which was found to climb steadily for 4 to 8 hr following cell activation (see Fig. 2 for an example of the time course of IL-3 mRNA induction).

IL-4 does not enhance HMC-1 growth

HMC-1 cells grow independently of added growth factors.[17] In the present study IL-4 at 10–50 ng/ml (24 hr) had no effect on mast numbers as assessed by direct cell counting and by measurement of ^{3}H-thymidine uptake. At 100 ng/ml IL-4 produced a modest but significant 10% fall in uptake of ^{3}H-thymidine ($P < 0{\cdot}001$ by Mann–Whitney U-test). IL-4 has been reported previously[10,19] to produce a similar degree of inhibition of HMC-1 growth to that seen here. Hence the up-regulation by IL-4 of transcription of IL-3 and IL-8 genes and secretion of their protein products does not reflect increases in HMC-1 cell number.

DISCUSSION

We report that recombinant human IL-4 enhances induced gene expression for IL-3 and IL-8 in a human mast cell line. IL-4 alone produced only a negligible effect on IL-3 and IL-8 gene expression in unstimulated cells, but enhanced expression of these genes quite markedly (by sixfold and fourfold respectively) when the cells were activated by a second stimulus, namely ionomycin. The enhancing effect of IL-4 is not a generalized effect but appears to be targeted at certain cytokine genes only: IL-4 had no effect on IL-4 mRNA in unstimulated mast cells and only weakly elevated IL-4 mRNA in activated cells; IL-4 was totally without effect on TNF-α mRNA in unstimulated and activated cells. We found too that IL-4 produced a far more dramatic enhancement of IL-3 than IL-8 protein secretion (15- to 20-fold compared to twofold). It appears that a sixfold increase in IL-3 gene transcription is accompanied by a disproportionate increase in secreted IL-3 product; whereas for IL-8, both mRNA and protein product are up-regulated to a similar extent. This may reflect a greater rate of translation of IL-3 mRNA compared to IL-8 mRNA in HMC-1 cells.

The resistance of TNF-α expression in HMC-1 cells to the regulatory effects of IL-4 suggests that this gene is under separate control from IL-3 and IL-8 in HMC-1 cells. TNF-α is to some extent stored preformed in rodent peritoneal mast cells,[5,20] human skin mast cells[7] and in HMC-1 cells;[10] in mouse mast cells the stored pool of TNF-α is released rapidly (within

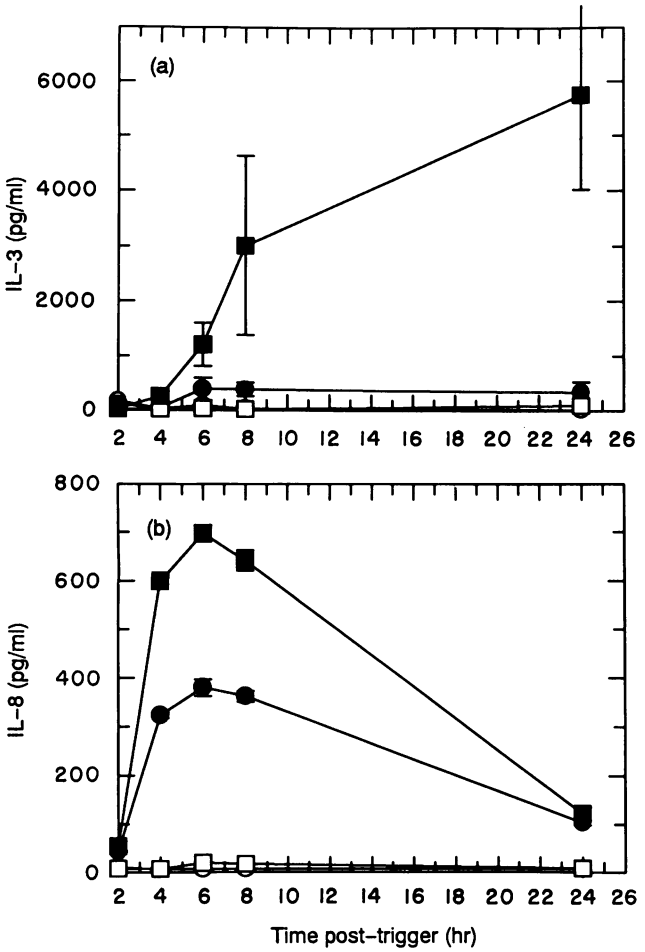

Figure 4. Time-dependent release of IL-3 (a) and IL-8 (b) from HMC-1 cells. Cells were cultured for 24 hr with or without IL-4, washed and challenged with ionomycin or control medium for 1 hr, then washed and returned to culture. Supernatant medium was removed for cytokine assays at the times shown. ○ = no IL-4, no ionomycin; ● = no IL-4, ionomycin-stimulated; □ = IL-4-treated, no ionomycin; ■ = IL-4-treated, ionomycin-stimulated. Results are means ± SD for four cytokine assays from two independent experiments.

10 min) after cell activation, while TNF-α released subsequently is *de novo* synthesized.[20] The relative refractoriness of the TNF-α gene in HMC-1 cells to up-regulation by IL-4 may be related to the fact that this gene appears to be constitutively expressed and not strongly induced on HMC-1 cell activation.

The cell line HMC-1, which is derived from a human mast cell leukemia,[17] resembles phenotypically a relatively immature mast cell: it expresses several characteristic mast cell molecules including the product of the proto-oncogene c-*kit* and the granule enzyme tryptase, but not a functional high affinity IgE receptor, and contains histamine at relatively low levels.[17,21] Cells of this line also express high affinity receptors for IL-4 (K_D = 0·04 to 0·2 nM).[19] In agreement with the present report, Sillaber *et al.*[10] failed to detect any effect of IL-4 on TNF-α expression, but did see IL-4 induced inhibition of IL-1β in these cells. The same group has reported that IL-4 enhances cell surface expression of the intercellular adhesion molecule ICAM-1[19] and inhibits expression of the c-*kit* product.[21] Considering the above, it is clear that IL-4 exerts diverse and complex regulatory effects on gene expression in mast cells, and by doing so may influence the functional role of mast cells in immune and inflammatory reactions. For example by promoting expression of IL-3, a cytokine important in activation of eosinophils,[22,23] and IL-8, a member of the *C–X–C* chemokine family important in neutrophil activation,[24] IL-4 may promote mast cell-dependent inflammatory reactions in which eosinophils and neutrophils are recruited and activated. At the same time IL-4 may promote mast cell adhesion to other cells via ICAM-1, while inhibiting responses mediated by mast cell IL-1β, and inhibiting also mast cell responsiveness to stem cell factor (the ligand at the c-*kit* product).

Whether IL-4 is an important regulator of cytokine gene expression in mast cells of tissue origin remains to be investigated. We know already that IL-4 promotes exocytotic secretion from mouse peritoneal tissue-type mast cells[16,25] and we postulate that this effect might extend to promotion of cytokine expression in these cells. We have also shown that exposure of mice to T-cell-activating chemical allergens leads to up-regulation *in vivo* of the secretory responsiveness of mast cells, and IL-4 may be implicated in this phenomenon.[26]

In conclusion, we have shown that IL-4 primes human mast cells for transcription of IL-3 and IL-8 genes and secretion of their protein products. IL-4, either of mast cell or lymphocyte origin, may induce amplification of mast cell-dependent processes involving IL-3 and IL-8, for example infiltration and activation of eosinophils and neutrophils in inflammation.

ACKNOWLEDGMENTS

We thank the Samuel Crossley–Barnes Fund for part support.

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