Regulation of 15-lipoxygenase expression in lung epithelial cells by interleukin-4

Roland BRINCKMANN*, Max S. TOPP†, Ildikó ZALÁN‡, Dagmar HEYDECK*, Peter LUDWIG*, Hartmut KÜHN*§, Wolfgang E. BERDEL† and Andreas J. R. HABENICHT‡

*Institute of Biochemistry, University Clinics Charité, Humboldt University, Hessische Strasse 3-4, 10115 Berlin, †Department of Internal Medicine, Universitätsklinikum Benjamin Franklin, Free University Berlin, Hindenburg Damm 30, 12200 Berlin, and ‡Clinic for Internal Medicine, Division of Endocrinology and Metabolism, University of Heidelberg, Bergheimer Strasse 58, 69115 Heidelberg, Federal Republic of Germany

We have studied the expression of the 15-lipoxygenase gene in various permanent mammalian cell lines in response to interleukins-4 and -13, and found that none of the cell lines tested expressed 5-, 12- or 15-lipoxygenase when cultured under standard conditions. However, when the lung carcinoma cell line A549 was maintained in the presence of either interleukin for 24 h or more, we observed a major induction of 15-lipoxygenase, as indicated by quantification of 15-lipoxygenase mRNA, by immunohistochemistry, by immunoblot analysis and by enzyme activity assays. This effect was 15-lipoxygenase-specific, since expression of 5- and 12-lipoxygenases remained undetectable. The time course of interleukin-4 treatment indicated maximal accumulation of both 15-lipoxygenase mRNA and functional protein after 48 h. Binding studies revealed that A549 cells express about 2100 high-affinity interleukin-4 binding sites per cell. The interleukin-4 mutant Y124D, which is capable of binding to the interleukin-4 receptor but is unable to trigger receptor activation, counteracted the effect of the wild-type cytokine. Other cell lines, including several epithelial cells and various monocytic cell lines expressing comparable numbers of interleukin-4 receptors, did not express 15-lipoxygenase when stimulated with interleukin-4. These data indicate that A549 cells selectively express 15-lipoxygenase when stimulated with interleukins-4 and -13. The activation of the interleukin-4/13 receptor(s) appears to be mandatory, but not sufficient, for 15-lipoxygenase gene expression.

INTRODUCTION

Among the members of the lipoxygenase family (EC 1.13.11.12), 15-lipoxygenase and the closely related leucocyte-type 12-lipoxygenases are of special interest, because of their ability to oxidize C_{18} fatty acids [1,2] and also more complex substrates such as phospholipids, biomembranes [2–4] and lipoproteins [5,6]. 15-Lipoxygenases are expressed at high levels during distinct stages of reticulocyte development [7], in human eosinophils [8], in human airway epithelial cells [9,10], and in macrophages of atherosclerotic lesions [11,12].

Although the biological function of 15-lipoxygenase still remains to be identified [1], its cell-specific expression pattern and its induction under distinctive differentiation conditions suggest tight, albeit complex, mechanisms of regulation. Immature reticulocytes, which have just left the bone marrow, contain high levels of 15-lipoxygenase mRNA in the form of translationally inactive ribonucleoprotein particles. Consequently, no functional 15-lipoxygenase protein is present at this stage of red cell maturation [13]. The lack of 15-lipoxygenase protein in the presence of high levels of 15-lipoxygenase mRNA has been ascribed to the presence of a translation inhibitor, termed lipoxygenase mRNA-binding protein, which binds to a 19 nt repeat motif in the 3′-untranslated region of 15-lipoxygenase mRNA [14]. At subsequent stages of maturation, translation of 15-lipoxygenase is initiated by yet to be identified processes, and the enzyme becomes one of the major reticulocyte proteins synthesized [15]. The appearance of 15-lipoxygenase in rabbit reticulocytes coincides with the breakdown of mitochondria and with the occurrence of specific lipoxygenase products in the cellular membranes [16]. These data, as well as *in vitro* maturation studies in the presence of lipoxygenase inhibitors [17], suggest that the enzyme is involved in the breakdown of intracellular membranes during reticulocyte maturation [17].

It has been reported that a functional 15-lipoxygenase is expressed at a high level in lipid-loaded macrophages of atherosclerotic lesions [11,12,18], and to a lesser extent in subtypes of arterial smooth muscle cells [19]. However, the role of this enzyme in the pathogenesis of cardiovascular disease is far from clear. Since circulating blood monocytes [20] or arterial smooth muscle cells of normal individuals do not express 15-lipoxygenase, the enzyme must be induced during foam cell formation, most probably in response to cytokines produced within the arterial wall. This assumption is supported by studies on the effects of cytokines on 15-lipoxygenase expression in cultured human blood monocytes [20,21] and in alveolar macrophages [22], in which interleukin-4 (IL-4) and IL-13 were identified as specific inducers of the enzyme. Although the intracellular signal transduction initiated by the activated IL-4 receptor has been studied in considerable detail [23–26] in various cells, the molecular mechanisms of IL-4/IL-13-mediated 15-lipoxygenase induction are not known.

The usefulness of primary cells for addressing the mechanistic questions of 15-lipoxygenase expression at the molecular level

Abbreviations used: IL-4, interleukin-4; CP-HPLC, chiral-phase HPLC; RP-HPLC, reverse-phase HPLC; SP-HPLC, straight-phase HPLC; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-PCR; 13-HODE, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; 15-HETE, 15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; DMEM, Dulbecco's modified Eagle's medium.

§ To whom correspondence should be addressed.

appears to be rather limited. Our attempts to study these mechanisms have been hampered by the biological variability of the cells and by their inherent impurity. To circumvent these problems we have initiated studies on permanent human haematopoietic and epithelial cell lines expressing the IL-4 receptor. Here we report that IL-4 and IL-13 trigger high-level expression of 15-lipoxygenase in the lung epithelial cell line A549 and that binding of the cytokines to the IL-4/IL-13 cell surface receptor(s) is required, but not sufficient, to induce 15-lipoxygenase gene expression.

EXPERIMENTAL

Materials

Human and murine recombinant IL-4 were obtained from UBI; recombinant IL-13 was from IC Chemicals (Ismaning, Germany). The IL-4 mutant Y124D was kindly provided by Dr. W. Sebald (University of Würzburg, Germany). Linoleic acid, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE) and 11*Z*,14*Z*-eicosadienoic acid were from Serva (Heidelberg, Germany). 15*S*-Hydroxy-11*Z*,13*E*-eicosadienoic acid, used as internal standard for the activity assays, was prepared from 11*Z*,14*Z*-eicosadienoic acid by reaction with soybean lipoxygenase, reduction of the hydroperoxide with triphenylphosphine and subsequent HPLC purification. *Taq* polymerase, avian myeloblastosis virus reverse transcriptase and immunochemicals were obtained from Boehringer (Mannheim, Germany). Human AB-serum was purchased from Sigma (Deisenhofen, Germany).

Cell lines were obtained from the following vendors: HL60 (human myeloblastic), U937 (human promyelomonocytic), THP1 (human monocytic) and MonoMac6 (human monocyte/macrophage) from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany); A549, HTB56, HTB54 (all human lung carcinoma), HTB43 (human squamous head and neck carcinoma), HTB38 (human colon carcinoma), J774 and P388.D1 (both mouse macrophage) from A.T.C.C. (Rockville, MD, U.S.A.). HMC1 cells (human mast cell) were a gift from Dr. T. Rosenbach (University of Berlin, Germany).

Cell culture and monocyte preparations

All cell lines were cultured according to the recommendations of the vendors unless stated otherwise. A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) human AB-serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). We tested different batches of fetal calf serum purchased from various vendors as a substituent for the human AB-serum, and found that 15-lipoxygenase could not be induced with some fetal calf serum samples. The reasons for this heterogeneity are unclear. However, with different batches of human AB-serum we always detected 15-lipoxygenase expression. For maximal lipoxygenase induction, IL-4 was added within 24 h after plating the cells. Cells were removed from the dishes by treatment with trypsin/EDTA, washed, resuspended in PBS and analysed for 15-lipoxygenase expression by reverse transcriptase-PCR (RT-PCR), immunohistochemistry, Western blot analysis and/or activity assays. For comparative studies, human blood monocytes were isolated from buffy coats by density gradient centrifugation and adherence to plastic dishes [20]. Monocytes were cultured in RPMI 1640 medium containing 10 % (v/v) fetal calf serum and antibiotics for 3 days in the absence or presence of cytokines. Cell viability (usually greater than 98 %) was determined by Trypan Blue exclusion.

Immunohistochemistry and immunoblots

Expression of 15-lipoxygenase was assessed by indirect immunofluorescence [20], using a polyclonal antibody (FPLC-purified IgG fraction) raised in guinea pigs against purified rabbit reticulocyte 15-lipoxygenase (primary antibody) and a fluorescein isothiocyanate-labelled goat anti-(guinea pig IgG) (secondary antibody). The anti-(rabbit 15-lipoxygenase) antibody showed strong cross-reactivity with human 15-lipoxygenase, but not with human 5-lipoxygenase or human platelet-type 12-lipoxygenase.

For Western blot analyses the cells were spun down, resuspended in 200 μl of lysis buffer (5 mM Tris/HCl, pH 7.5, with 1 mM EGTA) and sonicated on ice (ten times for 0.5 s at 40 W with a tip sonifier; Braun, Melsungen, Germany). Lysates were prepared for SDS/PAGE by mixing with 5-fold concentrated sample buffer (60 mM Tris/HCl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % mercaptoethanol) and boiling for 5 min. Aliquots of the homogenate were taken for protein determination. A sample of 40 μg of cellular protein was applied to electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Serva, Heidelberg, Germany) by semi-dry blotting and stained using the polyclonal anti-15-lipoxygenase primary antibody at a dilution of 1:500 and a horseradish peroxidase-conjugated anti-(guinea pig IgG) secondary antibody.

HPLC analyses of lipoxygenase products

For measurements of lipoxygenase activity, cells were trypsinized from the culture dishes, centrifuged for 8 min at 200 ***g*** and resuspended in PBS at a concentration of 5×10^6 cells/ml. After addition of linoleic acid or arachidonic acid (100 μM final concentration) the cells were sonicated on ice and the homogenates were incubated for 20 min at 37 °C. After addition of 13*S*-hydroxy-11*Z*,13*E*-eicosadienoic acid as internal standard and 2 vol. of ice-cold methanol, the protein precipitates were removed by centrifugation and aliquots were directly injected for reverse-phase HPLC (RP-HPLC) analysis. HPLC was carried out on a Shimadzu instrument coupled with a Hewlett Packard diode array detector 1040A. RP-HPLC was performed on a Nucleosil C-18 column (Macherey/Nagel, Düren, Germany; KS-system, 250 mm × 4 mm, 5 μm particle size). A solvent system of methanol/water/acetic acid (85:15:0.1, by vol.) and a flow rate of 1 ml/min were used. Chromatograms were quantified by peak areas. A calibration curve (five point measurements) for 13-HODE was established. The fractions containing the oxygenated polyenoic fatty acids were pooled, the solvent was evaporated, and the residue was reconstituted in a mixture of n-hexane/propan-2-ol/acetic acid (100:2:0.1, by vol.) and injected for straight-phase HPLC (SP-HPLC) and/or chiral-phase HPLC (CP-HPLC) analysis. SP-HPLC of the hydroxy fatty acid isomers was carried out on a Zorbax SIL column (Macherey/Nagel; KS-system, 250 mm × 4 mm, 5 μm particle size) with a solvent system consisting of n-hexane/propan-2-ol/acetic acid (100:2:0.1, by vol.) and a flow rate of 1 ml/min. CP-HPLC was carried out on a Chiralcel OD column (Diacel Chem.; 250 mm × 4.6 mm, 5 μm particle size; distributed by Baker, Gross-Gerau, Germany) with a solvent system of hexane/propan-2-ol/acetic acid (100:5:0.1, by vol.) and a flow rate of 1 ml/min. For detection of the hydroxy fatty acids, the absorbance at 235 nm was recorded.

Competitive RT-PCR

The quantification of 15-lipoxygenase mRNA in A549 cells was performed using competitive RT-PCR [27]. RNA was isolated using RNAzol® B (WAK Chemie, Bad Homburg, Germany)

[28]. Total RNA (3 μg) was reverse-transcribed at 37 °C for 90 min in 45 μl of 50 mM Tris/HCl buffer, pH 8.2, containing 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 100 μg/ml BSA, 30 units of RNase inhibitor, 0.166 mM of each dNTP, 150 pmol of oligo(dT) primer and 15 units of reverse transcriptase. Samples were heated to 95 °C for 10 min. For 15-lipoxygenase, the PCR primers 5′-GGGGCTGGCCGACC-TCGCTATC-3′ and 5′-TCCTGTGCGGGGCAGCTGGAGC-3′ were selected from regions displaying minimal sequence similarity to the sequences of human 12-lipoxygenase and leucocyte 5-lipoxygenase, and proved to be not suitable for the amplification of the human cDNAs of these two enzymes. The primers for the PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-TCGGAGTCAACGGATTTGG-TCGTA-3′ and 5′-ATGGACTGTGGTCATGAGTCCTTC-3′. Samples of 2 μl of the reverse transcription products were amplified in the presence of a range of known concentrations of internal standards (human 15-lipoxygenase cDNA with a 117 bp deletion and human GAPDH cDNA with a 105 bp deletion respectively). After initial denaturation for 3 min at 94 °C, PCR was carried out for 23–35 cycles. Each cycle consisted of a denaturing period (40 s at 94 °C), an annealing phase (30 s at 66 °C for GAPDH and at 71 °C for 15-lipoxygenase) and an extension period (30 s at 72 °C for both primer sets). The reaction mixture was 10 mM Tris/HCl buffer, pH 8.3, containing 50 mM KCl, 2 mM $MgCl_2$, 0.1 mg/ml gelatine, 6 pmol of primer sets, 100 μM of each dNTP, 100 μg/ml BSA and 2.5 units of *Taq* DNA polymerase. PCR products were separated by 2 % agarose gel electrophoresis. For quantification, DNA was stained with ethidium bromide and analysed densitometrically. Densitometric data were corrected for molar equivalence and plotted on a log/log scale as a function of internal-standard-derived PCR products.

IL-4 binding studies

The dissociation constants (K_d values) of IL-4 binding sites on lung carcinoma A549 and HTB56 cells were determined by Scatchard analyses of the specific binding of ^{125}I-labelled IL-4 [29]. At 24 h prior to the binding assays, adherent cells were plated in 6-well tissue culture plates at a density of 10^6 cells/well. The cells were washed twice with serum-free DMEM containing 1 mg/ml BSA, and ^{125}I-labelled IL-4 (10–500 pM) was added. Monolayers were incubated for 60 min at 37 °C and non-specific binding was determined after the addition of a 100-fold molar excess of unlabelled recombinant human IL-4. Cells were washed twice with 1 ml of ice-cold DMEM containing 1 mg/ml BSA and removed from the wells by lysis with 1 ml of 1 M NaOH. Radioactivity was counted using a Berthold (Freiburg, Germany) γ-radiation counter.

RESULTS

IL-4 triggers 15-lipoxygenase expression in the human lung epithelial cell line A549

Human peripheral monocytes do not express 15-lipoxygenase constitutively [20]. However, after maintaining the cells for 3 days in the presence of IL-4, significant 15-lipoxygenase expression can be detected [20]. When we analysed the 15-lipoxygenase content of human peripheral monocytes maintained for 3 days in the presence of IL-4 (67–2000 pM) by immunohistochemistry, we noted that, even at high IL-4 concentrations, not all monocytes were stained 15-lipoxygenase-positive. The proportion of lipoxygenase-positive cells varied between 10 and 70 % at 670 pM IL-4, and never reached 100 % even after longer incubation periods. Activity assays with various monocyte preparations from different donors indicated a similar variability (0.1–2.0 μg of 13-HODE formation/10^6 cells). These data suggest the presence of blood monocyte subpopulations that are unable to respond to IL-4 stimulation by expressing 15-lipoxygenase.

In order to avoid the problems of a heterogeneous cell population for our mechanistic studies, we screened several permanent haematopoietic and epithelial cell lines expressing the IL-4 receptor for 15-lipoxygenase expression by immunohistochemistry. If maintained under standard conditions no 15-lipoxygenase expression was detected in any of these cell lines. However, we observed apparent 15-lipoxygenase-positive staining in the human lung carcinoma cell line A549 if the cells were cultured for 5 days in the presence of IL-4 (Figure 1B). In contrast, their untreated counterparts did not show any fluorescence above background intensity (Figure 1A). As with monocytes, between 20 and 60 % of the A549 cells (different sets of experiments) were stained 15-lipoxygenase-positive after 5 days of IL-4 treatment. Thus there appears to be a similar heterogeneity in the cell population to that described for human peripheral monocytes. In addition to A549 cells, IL-4-treated human HTB38 colon carcinoma cells showed significant 15-lipoxygenase staining in about 1–5 % of the cells (results not shown).

Since immunohistochemistry may be misleading because of a possible cross-reactivity of the antibody with non-lipoxygenase proteins, enzyme activity assays were carried out. For this purpose, cell homogenates were incubated with exogenous substrate and the lipoxygenase products were analysed by RP-HPLC. Products co-migrating with authentic standards of 13-HODE were formed when homogenates of IL-4-treated A549 cells were incubated with linoleic acid (Figure 2, line B). Quantification of HPLC peak areas indicated a specific lipoxygenase activity of 0.542 ± 0.117 μg of 13-HODE/10^6 cells ($n = 6$). In contrast, no lipoxygenase products were found when A549 cells were cultured in the absence of IL-4 (Figure 2, line A). IL-13 also triggered 15-lipoxygenase expression (Figure 2, line C) in A549 lung epithelial cells (0.750 ± 0.042 μg of 13-HODE/10^6 cells; $n = 2$).

Since under our chromatographic conditions 13-HODE and 9-HODE were not separated, the products co-migrating with 13-HODE on RP-HPLC were prepared and further analysed for positional and enantiospecificity by SP-HPLC and CP-HPLC. We found that more than 90 % of the hydroxy fatty acid formed by IL-4- and IL-13-treated A549 cells co-migrated with 13-HODE on SP-HPLC (results not shown). Analysis of the enantiomer composition revealed a strong preponderance of the *S*-isomer in both cases (Figure 2, insets). When arachidonic acid was used as substrate, 15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HETE) was identified as the major product. These product patterns indicate the expression of an arachidonate 15-lipoxygenase and exclude non-enzymic lipid peroxidation as a source of the oxygenated fatty acids.

Monocytic cell lines and various epithelial cells do not up-regulate 15-lipoxygenase expression in response to IL-4

All cell lines screened by immunohistochemistry were re-examined for 15-lipoxygenase activity. As indicated in Table 1, none of the monocytic cell lines tested exhibited measurable 15-lipoxygenase activity either in the absence or in the presence of IL-4. Moreover, primary mixed human lymphocytes, the mast cell line HMC1, the two human lung carcinoma cell lines HTB54 and HTB56 and the human squamous head and neck carcinoma cell line HTB43 remained unresponsive. However, small amounts

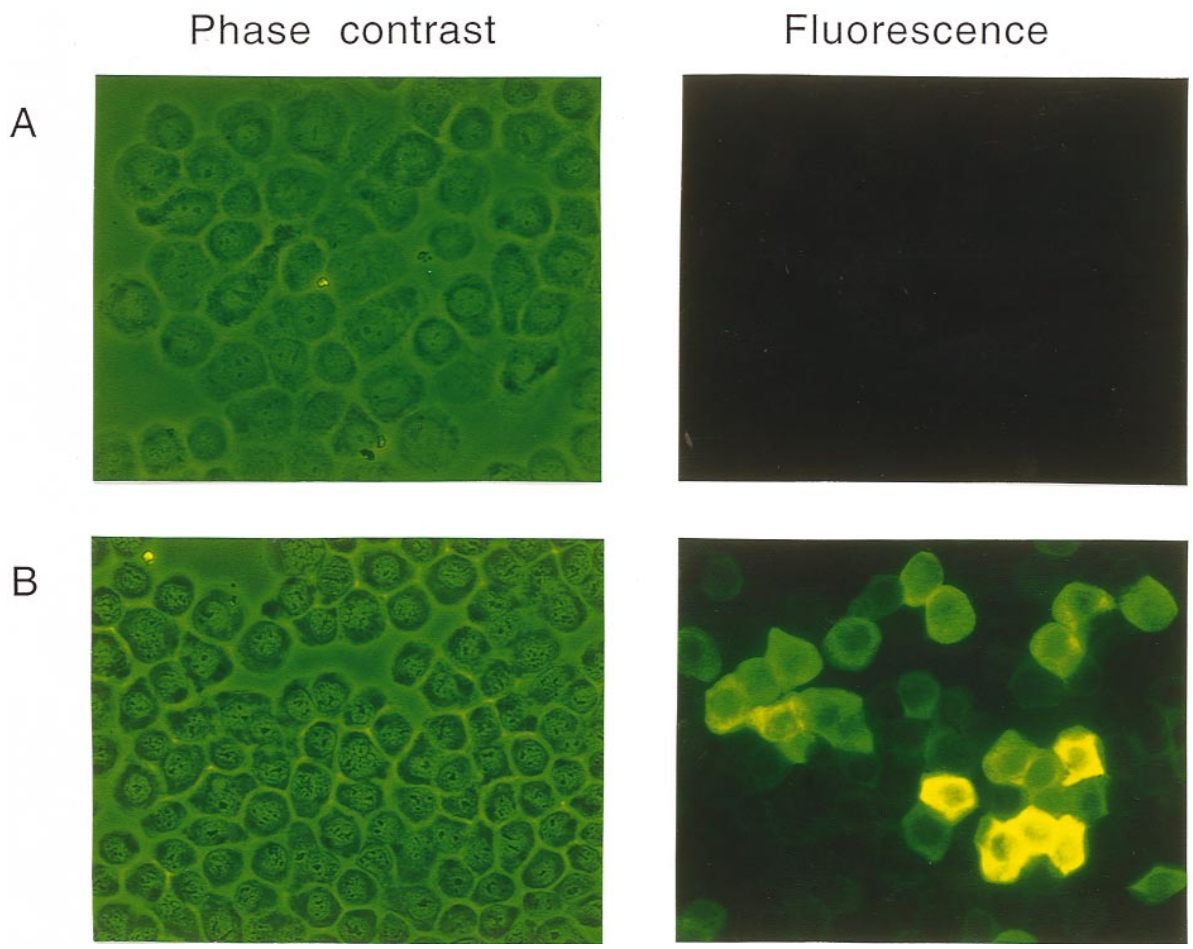

Figure 1 Immunohistochemical staining of 15-lipoxygenase in A549 cells

A549 cells were cultured for 120 h in the absence (**A**) or in the presence (**B**) of 670 pM IL-4. Cells were harvested by trypsin treatment and cytospin preparations were used for immunostaining as described in the Experimental section.

of 13-HODE (21 ng/10^6 cells) were detected when homogenates of the human colon carcinoma cell line HTB38 were incubated with linoleic acid. SP-HPLC revealed a 13-HODE/9-HODE ratio of about 2:1 (results not shown), and analysis of the enantiomer composition of the 13-HODE indicated a 2:1 preponderance of the *S*-enantiomer (Table 1). These data, which suggest the occurrence of small amounts of 15-lipoxygenase in IL-4-treated HTB38 cells, are in line with the immunohistochemical staining of this cell line.

Differential up-regulation of 15-lipoxygenase is not due to differences in expression of the IL-4 receptor

Activity assays and immunohistochemistry indicated the expression of 15-lipoxygenase when A549 cells were stimulated with IL-4, whereas other epithelial cells, including the human lung carcinoma cell lines HTB54 and HTB56, did not express the enzyme when cultured under identical conditions. To exclude the possibility that this unresponsiveness was due to different expression levels of the IL-4 cell surface receptor, we performed comparative IL-4 binding studies with A549 (lipoxygenase-positive) and HTB56 (lipoxygenase-negative) cells. Both cell types were found to express comparable numbers of specific IL-4 binding sites per cell. Scatchard-plot analyses of the binding data indicated the presence of 2100 binding sites per A549 cell and 1700 binding sites per HTB56 cell (Figures 3A and 3B). The K_d values of 55 pmol/l (A549) and 30 pmol/l (HTB56) were of the same order of magnitude as the corresponding value for the high-affinity IL-4 receptor on human monocytes [30]. Various literature reports indicate that most of the cell lines tested in the present study express a functional IL-4 cell surface receptor (Table 1). These data, and the inability of most cells to respond with 15-lipoxygenase expression when stimulated with IL-4, suggest that the activation of the IL-4-induced signal transduction cascade does not necessarily lead to 15-lipoxygenase expression.

Activation of the IL-4 receptor is required, but is not sufficient, for up-regulation of 15-lipoxygenase in A549 cells

The specificity of the cytokine-mediated 15-lipoxygenase induction in monocytes [20] suggested that IL-4 cell surface receptors may be involved. In order to exclude non-receptor-mediated mechanisms, competition studies with the IL-4 mutant Y124D were carried out. This mutant binds to the IL-4 receptor with a comparable affinity as the wild-type IL-4, but is unable to

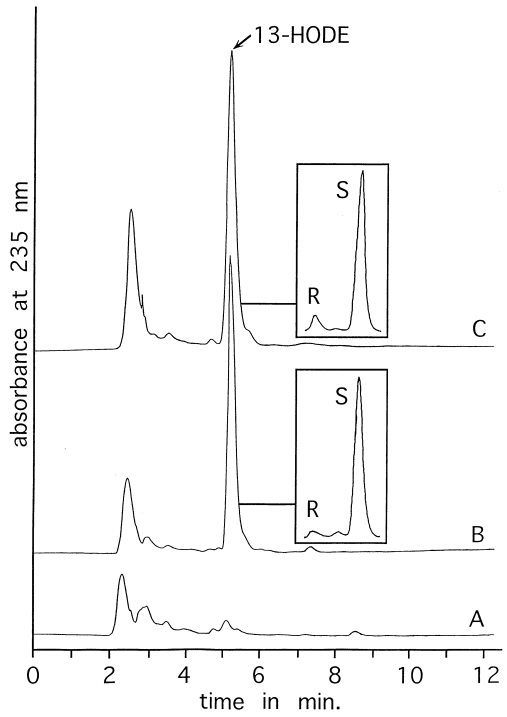

Figure 2 Formation of 15-lipoxygenase products by A549 cells treated with IL-4 and IL-13

A549 cells were cultured for 5 days in the absence (line A) or in the presence (line B) of 670 pM IL-4, or in the presence of 670 pM IL-13 (line C). After harvesting, the cells were resuspended in 1 ml of PBS, disintegrated by sonication and incubated for 20 min at 37 °C with 100 μM linoleic acid. Product preparation and HPLC analysis were carried out as described in the Experimental section. Insets: analysis of the enantiomer composition of the major oxygenation product 13-HODE by CP-HPLC.

trigger the signal transduction cascade [31,32]. We found that Y124D was unable to induce 15-lipoxygenase in A549 cells and in human blood monocytes at concentrations up to 13.4 nM (results not shown). However, Y124D did act as competitor for wild-type IL-4, as indicated by the inhibition of 15-lipoxygenase expression (Figure 4).

The time course of 15-lipoxygenase mRNA expression is paralleled by enzyme activity

In immature rabbit reticulocytes, the expression of functional 15-lipoxygenase is prevented at the translational level by the binding of a regulatory protein to the mRNA [14]. In these cells no 15-lipoxygenase protein is present, although a high concentration of 15-lipoxygenase mRNA was detected [13]. In order to find out whether similar processes occur in IL-4-treated A549 cells, competitive RT-PCR of 15-lipoxygenase mRNA, immunoblots of 15-lipoxygenase protein and 15-lipoxygenase activity assays were carried out in parallel. Immunoblot analyses (Figure 5) of the cell lysates indicated that cells cultured in the absence of IL-4 for up to 120 h did not show a 15-lipoxygenase signal (lane A). However, when the cells were maintained in the presence of IL-4 for 24 h a lipoxygenase signal appeared (lane B). This signal was even stronger after a 144 h culture period (lane C). With quantitative RT-PCR we were unable to detect significant amounts of 15-lipoxygenase mRNA in cells maintained for up to 144 h in the absence of IL-4, even after extended PCR cycles (results not shown). However, when the cells were cultured in the presence of IL-4, a time-dependent increase in 15-lipoxygenase mRNA levels was observed. 15-Lipoxygenase mRNA reached significant levels above background after 6 h of IL-4 treatment. At this time point we calculated a value of approx. 25 copies of 15-lipoxygenase mRNA per 10^6 GAPDH transcripts (results not shown). After 24 h, approx. 630 copies of 15-lipoxygenase

Table 1 Induction of 15-lipoxygenase by IL-4 in various mammalian cells

Cells were maintained in the presence or absence of IL-4 (670 pM final concentration) for the times indicated. After harvesting, the cells were resuspended in 1 ml of PBS, disintegrated by sonication and incubated with 100 μM linoleic acid for 20 min at 37 °C. After acidification, the lipids were extracted and analysed for hydroxy fatty acids by RP-HPLC. Values in parentheses indicate the relative proportions of *S*- and *R*-enantiomers. ↑, Up-regulation; ↓, down-regulation. ICAM, intracellular cell adhesion molecule. Data obtained from the references indicated.

Cell type	IL-4 exposure (days)	13-HODE (ng/10^6 cells)		Il-4 receptor expression	Selected biological effects of IL-4
		−IL-4	+IL-4 (*S*/*R*)		
Human peripheral monocytes	3	< 10	2170 (89:11)	Yes [39]	CD23 ↑ [48]
Human lymphocytes	3	< 10	< 10	Yes [31]	Proliferation ↑ (T-cells) [31] CD23 ↑ (B-cells) [31]
HL60 (human myeloblastic/promyelocytic)	3	< 10	< 10	Yes [39]	Proliferation ↓ [45]
U937 (human promyelomonocytic)	3	< 10	< 10	Yes [39]	CD23 ↑ [46]
THP1 (human monocytic)	3	< 10	< 10	Yes [38]	CD23 ↑ [46]
MonoMac6 (human monocytic)	5	< 10	< 10	Yes (α-chain) [44]	CD14 ↓ [43]
J774 (murine macrophage)	5*	< 10	< 10	Yes [41]	Oxidative burst ↑ [47]
P388.D1 (murine macrophage)	3*	< 10	< 10	Yes [40]	†
HMC1 (human mast cell)	3	< 10	< 10	Yes [42]	ICAM-1 ↑ [42]
A549 (human lung carcinoma)	5	< 10	542 (91:9)	Yes (present study)	Proliferation ↓ [50]
HTB56 (human lung carcinoma)	5	< 10	< 10	Yes (present study)	Proliferation ↓ [50]
HTB54 (human lung carcinoma)	5	< 10	< 10	†	†
HTB43 (human squamous head and neck carcinoma)	5	< 10	< 10	Yes [49]	Proliferation ↓ [51]
HTB38 (human colon carcinoma)	5	< 10	21 (65:35)	Yes [49]	Proliferation ↓ [51]

* Murine recombinant IL-4.
† No data available.

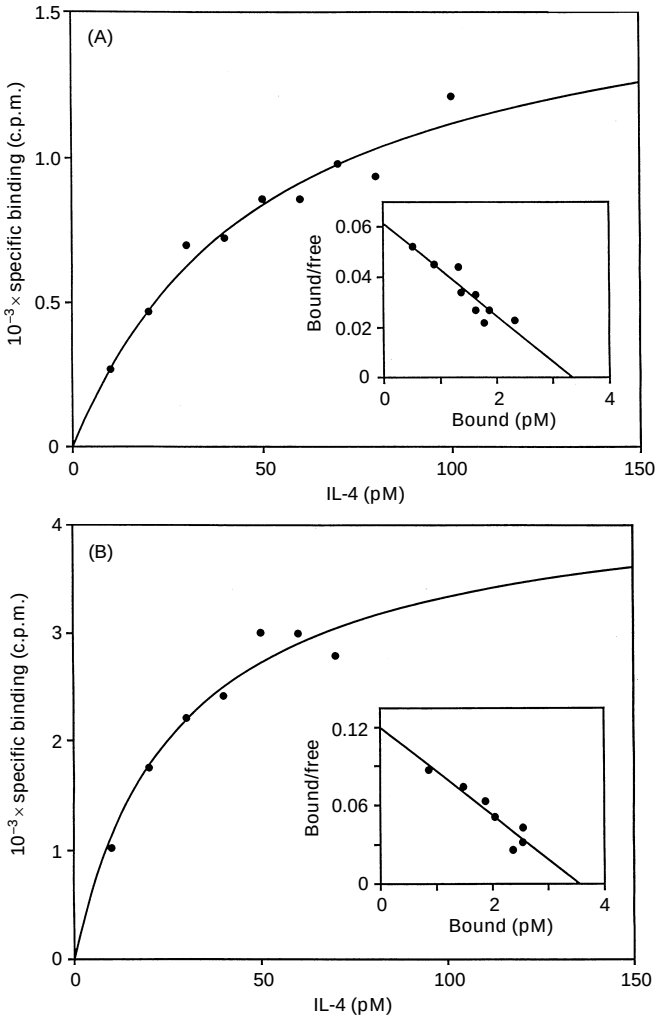

Figure 3 Specific binding of ^{125}I-labelled IL-4 to A549 cells and HTB56 cells

A549 cells (**A**) and HTB56 cells (**B**) were incubated in the presence of the indicated concentrations of ^{125}I-labelled IL-4. Scatchard analyses of the binding data are shown in the insets. Correlation coefficients for linear regressions were −0.92 and −0.94 for A549 and HTB56 cells respectively.

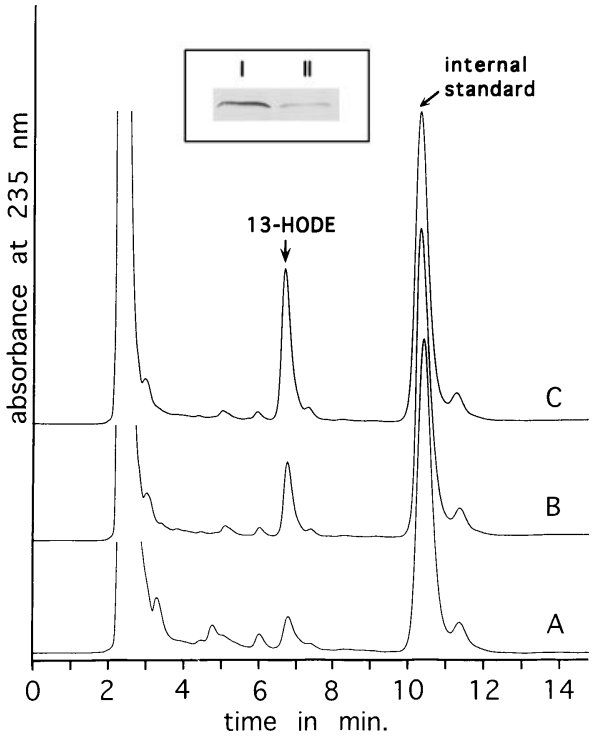

Figure 4 The IL-4 mutant Y124D inhibits IL-4-induced 15-lipoxygenase expression

A549 cells were cultured for 5 days under the conditions specified below. After harvesting, cells were resuspended in 1 ml of PBS and aliquots were prepared for Western blot analysis (inset). Homogenates prepared from the remaining cells were incubated with 100 μM linoleic acid, and the lipoxygenase products were analysed by RP-HPLC as described in the Experimental section. A, Cells cultured for 5 days in the absence of cytokines; B, cells cultured for 5 days in the presence of 670 pM IL-4 and 13.4 nM IL-4 mutant Y124D; C, cells cultured for 5 days in the presence of 670 pM IL-4. Inset: lane I, immunoblot of sample C; lane II, immunoblot of sample B.

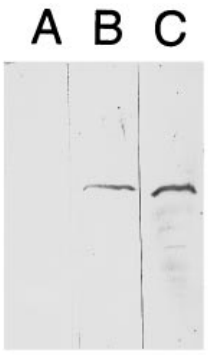

Figure 5 Western blot analysis of 15-lipoxygenase protein

A549 cells were cultured in the absence or in the presence of 670 nM IL-4 for various time periods. Cells were harvested and prepared for the immunoblots as described in the Experimental section. Lane A, cells cultured for 5 days in the absence of IL-4; lane B, cells cultured for 24 h in the presence of IL-4; lane C, cells cultured for 5 days in the presence of IL-4.

mRNA/10^6 GAPDH transcripts were detected, reaching a maximum in the time course of IL-4 treatment of about 1500 copies per 10^6 GAPDH transcripts after 48 h. In Figure 6 the time courses of both the relative 15-lipoxygenase mRNA concentration and the 15-lipoxygenase activity during a culture period of 144 h are shown. It can be seen that the increase in 15-lipoxygenase mRNA is paralleled by enzyme activity, suggesting that there may not be a time-dependent down-regulation of the translation of 15-lipoxygenase mRNA by regulatory proteins as occurs in rabbit reticulocytes [14].

IL-4 induces 15-lipoxygenase but not the expression of other lipoxygenases

Using specific primers for human platelet 12-lipoxygenase and human leucocyte 5-lipoxygenase, we did not detect significant amounts of 5- or 12-lipoxygenase transcripts in A549 cells even when they were cultured for up to 5 days in the presence of IL-4. Moreover, we were unable to detect the formation of significant amounts of 5-HETE in our activity assays. These data suggest that IL-4 induces 15-lipoxygenase synthesis, but not the expression of 5- or 12-lipoxygenase, in A549 cells.

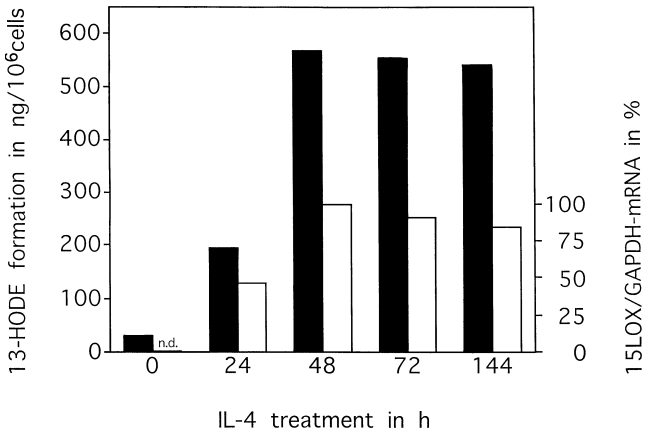

Figure 6 Time courses of 15-lipoxygenase mRNA expression and 15-lipoxygenase activity

A549 cells were maintained in the presence of 670 pM IL-4 for the times indicated. 15-Lipoxygenase activity and mRNA content were determined as described in the Experimental section. Each column represents the mean of at least two independent determinations. ■, 15-Lipoxygenase activity (13-HODE formed/10^6 cells). □, Cellular 15-lipoxygenase (15LOX) mRNA content, expressed as a percentage of the maximal mRNA level reached after 48 h (100% represents 1514 copies of 15-lipoxygenase mRNA/10^6 GAPDH transcripts); n.d., not detectable.

DISCUSSION

The closely related cytokines IL-4 and IL-13 have been reported previously to induce 15-lipoxygenase expression in human peripheral monocytes and alveolar macrophages [20–22]. Here we report for the first time that both cytokines may exhibit a similar effect in replicating extra-haematopoietic cells. We found that the accumulation of 15-lipoxygenase mRNA is paralleled by the appearance of the functional enzyme, and that the induction is 15-lipoxygenase-specific since the expression of both platelet-type 12-lipoxygenase and leucocyte 5-lipoxygenase were not affected. Since A549 cells are a permanent cell line, and since the expression level of 15-lipoxygenase is comparable with that in IL4-treated monocytes (up to 600 ng of HODE formed/10^6 cells), these cells may constitute a more suitable model in which to study the mechanism of cytokine-induced 15-lipoxygenase expression. The experimental finding that only the human 15-lipoxygenase, but not the 12- or 5-lipoxygenases, is induced by IL-4/IL-13 clearly indicates that these enzymes are differentially regulated.

The mechanism of IL-4/IL-13-induced signal transduction leading to 15-lipoxygenase expression in mammalian cells remains unclear. Nuclear run-on transcription assays in human blood monocytes suggested that the enzyme may be regulated primarily by stabilizing its mRNA [33]. These data do not exclude elements of transcriptional regulation during IL-4-induced 15-lipoxygenase expression in A549 cells. It is well established that IL-4 activates specific transcription factors [34] which bind to IL-4-responsive elements in the promotor region of respective inducible genes. However, recent studies on the transcriptional regulation of rabbit 15-lipoxygenase were unsuccessful in identifying positive regulation sites of transcription. Instead, transcriptional silencer regions have been detected which are functional in several extra-haematopoietic cells [35].

The cytokine-specific induction of 15-lipoxygenase in A549 cells and the competition studies with the IL-4 mutant Y124D suggest an involvement of specific cell surface receptors. As reported previously, IL-4 and IL-13 show cross-binding between IL-4 and IL-13 receptors [36]. Thus our data do not allow a statement about whether the IL-4 receptor, the structurally related IL-13 receptor or both are involved in cytokine-triggered 15-lipoxygenase expression. Several intracellular second messenger pathways have been reported to be activated by agonist binding to the IL-4 receptor [23–26]. It remains to be established which of these pathways, if any, is implicated in IL-4-induced 15-lipoxygenase expression and whether the recently identified IL-4 specific transcription factor IL-4 Stat [34] may be involved.

As indicated in Table 1, we were unable to induce significant 15-lipoxygenase expression in any of the 14 human cell lines tested except for A549 cells. The reasons for these results are not immediately apparent, since most of the cell lines tested express the IL-4 receptor and/or are capable of responding to IL-4 treatment [37–49]. These findings suggest the existence of cell-specific signalling pathways leading to 15-lipoxygenase expression in selected cell types. It will be a challenge for future work to identify components of such pathways in A549 cells which are apparently lacking in other epithelial and haematopoietic cells.

There are several potential implications of IL-4-dependent 15-lipoxygenase expression in A549 cells. The biology of IL-4 appears to be rather complex [50,51]. It is produced by T_{H2} helper cells, by mast cells and by basophilic granulocytes [52]. One may assume that mast cells, which are involved in inflammatory and allergic lung disease, participate in the regulation of 15-lipoxygenase gene expression in the airway epithelial cells [53]. In fact 15-HETE, which is the major arachidonic acid oxygenation product in human bronchi [54], was shown to potentiate the allergen-induced release of allergic mediators [55]. In this context, it is of particular interest to note that transgenic mice overexpressing IL-4 develop a disorder which is associated with alterations in mast cell counts and which resembles human allergic disease [56]. It remains to be examined whether 15-lipoxygenase is involved in these processes.

This work was supported by grants from the Deutsche Forschungsgemeinschaft to H.K. (Ku 961/1-1) and A.J.R.H. (Ha 1083/8-2), and in part by a grant (PL 93/1790; H.K. coordinator) from the European Community in the framework of the BIOMED1 program.

REFERENCES

1 Yamamoto, S. (1992) Biochim. Biophys. Acta **1128**, 117–131
2 Takahashi, Y., Glasgow, W. C., Suzuki, H., Taketani, Y., Yamamoto, S., Anton, M., Kühn, H. and Brash, A. R. (1993) Eur. J. Biochem. **218**, 165–171
3 Murray, J. J. and Brash, A. R. (1988) Arch. Biochem. Biophys. **265**, 514–523
4 Kühn, H., Belkner, J., Wiesner, R. and Brash, A. R. (1990) J. Biol. Chem. **265**, 18351–18361
5 Belkner, J., Wiesner, R., Rathmann, J., Barnett, J., Sigal, E. and Kühn, H. (1993) Eur. J. Biochem. **213**, 251–261
6 Kühn, H., Belkner, J., Suzuki, H. and Yamamoto, S. (1994) J. Lipid Res. **35**, 1749–1759
7 Rapoport, S. M., Schewe, T., Wiesner, R., Halangk, W., Ludwig, P., Janicke-Höhne, M., Tannert, C., Hiebsch, C. and Klatt, D. (1979) Eur. J. Biochem. **95**, 545–561
8 Turk, J., Maas, R. L., Brash, A. R., Roberts, L. J. and Oates, J. A. (1982) J. Biol. Chem. **257**, 7068–7076
9 Nadel, J. A., Conrad, D. J., Ueki, I. F., Schuster, A. and Sigal, E. (1991) J. Clin. Invest. **87**, 1139–1145
10 Holtzmann, M. J. (1988) Biochim. Biophys. Acta **963**, 401–413
11 Yla-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Glass, C. K., Sigal, E., Sarkioia, T., Witztum, J. T. and Steinberg, D. (1991) J. Clin. Invest. **87**, 1146–1152
12 Yla-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Glass, C. K., Sigal, E., Witztum, J. T. and Steinberg, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6959–6963
13 Thiele, B. J., Andree, H., Höhne, M. and Rapoport, S. M. (1982) Eur. J. Biochem. **129**, 133–141
14 Ostareck-Lederer, A., Ostareck, D. H., Standart, N. and Thiele, B. J. (1994) EMBO J. **13**, 1476–1481

15 Thiele, B. J., Belkner, J., Andree, H., Rapoport, T. A. and Rapoport, S. M. (1979) Eur. J. Biochem. **96**, 563–569
16 Kühn, H. and Brash, A. R. (1990) J. Biol. Chem. **265**, 1454–1458
17 Rapoport, S. M. and Schewe, T. (1986) Biochim. Biophys. Acta **864**, 471–495
18 Kühn, H., Belkner, J., Zaiss, S., Fährenklemper, T. and Wohlfeil, S. (1994) J. Exp. Med. **179**, 1903–1911
19 Hugou, I., Blin, P., Henri, J., Daret, D. and Larrue, J. (1995) Atherosclerosis **113**, 189–195
20 Conrad, D. J., Kühn, H., Mulkins, M., Highland, E. and Sigal, E. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 217–221
21 Nassar, G. M., Morrows, J. D., Roberts, II, L. J., Fadi, F. G. and Badr, K. F. (1994) J. Biol. Chem. **269**, 27631–27634
22 Levy, B. D., Romano, M., Chapman, H. A., Reilly, J. J., Drazen, J. and Serhan, C. N. (1993) J. Clin. Invest. **92**, 1572–1579
23 Keegan, A. D., Johnston, J. A., Tortolani, P. J., McReynolds, L. J., Kinzer, C., O'Shea, J. J. and Paul, W. E. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7681–7685
24 Pernis, A., Witthuhn, B., Keegan, A. D., Nelms, K., Garfein, E., Ihle, J. N., Paul, W. E., Pierce, J. H. and Rothman, P. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7971–7975
25 Izuhara, K., Yang, G., Miyajima, A., Howard, M. and Harada, N. (1993) Res. Immunol. **144**, 584–590
26 Arruda, S. and Ho, J. K. (1992) J. Immunol. **149**, 1258–1264
27 Gilliland, G., Perrin, S., Blanchard, K. and Bunn, H. F. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2725–2729
28 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
29 Toi, M., Harris, A. L. and Bicknell, R. (1991) Biochem. Biophys. Res. Commun. **174**, 1287–1293
30 Wagteveldt, A. J., van Zanten, A. K., Esselink, M. T., Halie, M. R. and Vellenga, E. (1991) Leukemia **5**, 782–788
31 Kruse, N., Tony, H. P. and Sebald, W. (1992) EMBO J. **11**, 3237–3244
32 Tony, H. P., Shen, B. J., Reusch, P. and Sebald, W. (1994) Eur. J. Biochem. **225**, 659–666
33 Conrad, D. J., Sigal, E. and Vanderbilt, J. (1994) 9th Int. Conf. Prostaglandins Relat. Compounds, Florence, June 6–10, abstract book, p. 39
34 Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M. and McKnight, S. L. (1994) Science **265**, 1701–1706
35 O'Prey, J. and Harrison, P. R. (1995) Nucleic Acids Res. **23**, 3664–3672
36 Zurawski, S. M., Vega, F., Huyghe, B. and Zurawski, G. (1993) EMBO J. **12**, 2663–2670
37 Vita, N., Lefort, S., Laurent, P., Caput, D. and Ferrara, P. (1995) J. Biol. Chem. **270**, 3512–3517
38 Park, L. S., Friend, D., Sassenfeld, H. M. and Urdal, D. L. (1987) J. Exp. Med. **166**, 476–488
39 Park, L. S., Friend, D., Grabstein, K. and Urdal, D. L. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1669–1673
40 Lowenthal, J. W., Castle, B. E., Christiansen, J., Schreurs, J., Rennick, D., Arai, N., Hoy, P., Takebe, Y. and Howard, M. (1988) J. Immunol. **140**, 456–464
41 Valent, P., Bevec, D., Maurer, D., Besemer, J., Di-Padova, F., Butterfield, J. H., Speiser, W., Majdic, O., Lechner, K. and Bettelheim, P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3339–3342
42 Quentmeier, H., Kolsdorf, K., Zaborski, M. and Drexler, H. G. (1994) Leukemia **8**, 1551–1556
43 Quentmeier, H., Kolsdorf, K., Zaborski, M. and Drexler, H. G. (1995) Leukemia **9**, 336–340
44 Vassiliades, S., Kyrpides, N. and Papamatheakis, J. (1992) Leuk. Lymphoma. **7**, 235–242
45 van Hal, P. T., Hopstaken-Broos, J. P., Wijkhuijs, J. M., Te-Velde, A. A., Figdor, C. G. and Hoogsteden, H. C. (1992) J. Immunol. **149**, 1395–1401
46 Tan, H. P., Nehlsen-Cannarella, S. L., Garberoglio, C. A. and Tosk, J. M. (1991) J. Leukocyte Biol. **49**, 587–591
47 Vercelli, D., Jabara, H. H., Lee, W., Woodland, N., Geha, R. S. and Leung, D. Y. M. (1988) J. Exp. Med. **170**, 1406–1416
48 Topp, M. S., Papadimitriou, C. A., Eitelbach, F., Koenigsmann, M., Oelmann, E., Koehler, B., Oderberg, D., Reuffi, B., Stein, H., Thiel, E. and Berdel, W. E. (1995) Cancer Res. **55**, 2173–2176
49 Topp, M. S., Koenigsmann, M., Mire-Sluis, A., Oberberg, D., Eitelbach, F., von Marshall, Z., Notter, M., Reufi, B., Stein, H., Thiel, E. and Berdel, W. E. (1993) Blood **82**, 2837–2844
50 Paul, W. E. (1991) Blood **77**, 1859–1870
51 Beckmann, M. P., Cosman, D., Fanslow, W., Maliszewski, C. R. and Lyman, S. D. (1992) Chem. Immunol. **51**, 107–134
52 Peyron, E. and Banchereau, J. (1994) Eur. J. Dermatol. **4**, 181–188
53 Sigal, E. and Conrad, C. D. (1995) Adv. Prostaglandin Thromboxane Leukotriene Res. **22**, 309–316
54 Hunter, J. A., Finkbeiner, W. E., Nadel, J. A., Goetzl, E. J. and Holtzman, M. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4633–4637
55 Lai, C. K. W., Polosa, R. and Holgate, S. T. (1990) Am. Rev. Respir. Dis. **141**, 1423–1427
56 Tepper, R. I., Levinson, D. A., Stanger, B. Z., Campos, T. J., Abbas, A. K. and Leder, P. (1990) Cell **62**, 457–467

Received 11 March 1996/17 April 1996; accepted 30 April 1996