Production of Interleukin-6 by Human Mast Cells and Basophilic Cells

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Since mast cells and basophils are thought to play a central role in several types of cutaneous inflammatory and allergic reactions, and since interleukin-6 (IL-6) is an important mediator in these processes, we have studied the ability of the human mast cell line HMC-1, the human basophilic cell line KU812, and human skin mast cells to produce IL-6. All three cell types proved to be potent sources of this cytokine after appropriate stimulation. Transcription of IL-6 mRNA was first detectable 2 h after stimulation with the ester phorbol myristate acetate (PMA) and the calcium ionophore A23187 in both cell lines, as evidenced by semiquantitative reverse transcriptase polymerase chain reaction analysis. Whereas resting cells did not produce IL-6 protein, PMA/A23187-stimulated cells released immunoreactive and biologically active IL-6, as demonstrated and quantitated by enzyme-linked immunosorbent assay and by the use of TEPC 1033 cells, an IL-6-dependent murine plasmacytoma cell line. Stimulated KU812 cells secreted sevenfold more IL-6 (up to 15 ng/ml) than HMC-1 cells (up to 2.4 ng/ml). Immunoblotting of HMC-1- and KU812 cell-derived IL-6 revealed several IL-6 forms in the molecular weight range of 21 to 30 kDa. Immunoelectron microscopic studies of human skin biopsies provided evidence that unstimulated mast cells do not contain preformed IL-6 but accumulate IL-6 in cytoplasmic and extruded granules after IgE-dependent stimulation. These findings suggest that IL-6 secreted by human mast cells and basophils potentially contributes to allergic, other immunologically mediated and nonspecific inflammatory responses. *Key words: basophils. J Invest Dermatol 106:75–79, 1996*

The role of mast cells and basophils as primary effector cells in IgE-dependent immediate hypersensitivity is well established. The finding that activated mast cells are also a source of several cytokines suggests an additional role of mast cells in late-phase reactions and other persistent inflammatory processes (for review see [1]). Among the cytokines known to be produced by rodent mast cells, interleukin-6 (IL-6), a pleiotropic cytokine with central functions in host response to injury and infection, is of particular importance. IL-6 elicits production of hepatic acute phase plasma proteins, induces differentiation, activation, and/or proliferation of B cells, T cells, and macrophages, and enhances IL-4-dependent IgE production [2] and the formation of IL-3-dependent multipotential colonies in hematopoietic stem cells (for review see [3,4]). IL-6 is produced in various phosphoglycosylated forms by monocytes/macrophages, B cells, T cells, fibroblasts, endothelial cells, and keratinocytes in response to cytokines, viruses, or endotoxin [3,4]. Moreover, *in vitro* production of IL-6 has been demonstrated in activated murine mast cell lines and in primary murine bone marrow mast cell cultures [5,6] as well as in activated rat peritoneal mast cells [7].

IL-6 is thought to be involved in several diseases including autoimmune disorders and plasma cell neoplasias and especially in inflammatory processes of the skin as diverse as scleroderma [8,9], psoriasis [10], and delayed pressure urticaria [11]. Also, a contribution of mast cells and basophils to the pathogenesis of fibrotic and/or inflammatory skin diseases such as scleroderma [12], psoriasis [13], and bullous pemphigoid [14,15] has been suggested. This coincidence of changes in mast cell and basophil numbers and the elevations in IL-6 serum levels in several of these diseases led us to investigate the ability of human mast cells and basophilic cells to produce IL-6 in cell cultures and in skin biopsies.

MATERIALS AND METHODS

Cell Culture HMC-1, an immature human mast cell line [16], was maintained in Iscove's medium (Seromed, Berlin, Germany), supplemented with 10% FCS (Seromed) and 10^{-5} M monothioglycerol (Sigma, Deisenhofen, Germany). KU812, a human early basophilic leukocyte cell line [17], was maintained in RPMI 1640 (Seromed)/15% fetal bovine serum. Human fibroblasts were isolated from foreskin and cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum. All cell culture media contained 2 mM glutamine (Seromed) and antibiotics (Seromed). One million cells per milliliter serum-free medium were stimulated with phorbol myristate acetate (PMA) and/or A23187 (both from Sigma) at various concentrations and for various time periods. Preconfluent fibroblast cultures were stimulated with 1 ng/ml IL-1β (specific activity: 5×10^8 units/mg in the A375 growth inhibition assay) (Genzyme, Boston, MA) for 24 h in serum-free Dulbecco's modified Eagle's medium.

Manuscript received December 30, 1994; revised August 24, 1995; accepted for publication August 29, 1995.

This work was presented in part at the Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung, Würzburg, Germany, November 18–20, 1994.

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Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; rIL-6, recombinant interleukin-6.

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Enzyme-Linked Immunosorbent Assay for IL-6 The amount of IL-6 protein in cell culture supernatants of HMC-1 or KU812 cells was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using a polyclonal goat anti-human IL-6 antibody (British Biotechnology, Oxford, U.K.) for coating and a monoclonal mouse anti-human IL-6 antibody (Hiss Diagnostics, Freiburg, Germany) and a biotinylated goat anti-mouse IgG $F(ab)_2$ fragment (Medac, Hamburg, Germany) for detection. Absorption of the streptavidin-alkaline phosphatase color reaction was measured at 450 nm and compared with serial dilutions of human recombinant IL-6 (rIL-6) (specific activity: 1 B9 unit/pg) (Hiss Diagnostics) as a standard. The lower detection limit was approximately 30 pg/ml. The ELISA did not cross-react with other known cytokines such as IL-1α and IL-1β, IL-2, tumor necrosis factor–α or granulocyte-macrophage colony–stimulating factor.

Bioassay for IL-6 The IL-6 bioassay was performed by measuring the [^{3}H]thymidine incorporation rates of the IL-6–dependent mouse plasmacytoma cell line TEPC 1033, as previously described [18]. The IL-6 content of cell culture supernatants was quantified in comparison to serial dilutions of human rIL-6 (Hiss Diagnostics). In order to prove specificity, culture supernatants were preincubated with a neutralizing goat anti-human IL-6 antibody (1 μg/ml) (British Biotechnology) or with equal amounts of nonimmune goat IgG (Dianova) in two experiments.

Polymerase Chain Reaction Analysis Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed as previously described [19]. Briefly, 3 μg of total cellular RNA, purified by the guanidium thiocyanate/cesium chloride method, was transcribed into cDNA using random priming. For comparison of IL-6 mRNA levels in different samples, cDNAs were first adjusted to equal concentrations of β-actin by the use of a β-actin control fragment [19] and were then analyzed for their content of IL-6 mRNA. The specificity of the amplification products was verified by restriction analysis with two enzymes indicative of the expected amplified sequence (data not shown). To control for contaminations, the polymerase chain reaction (PCR) mixture was amplified without cDNA or contained RNA that had not been reverse transcribed. Primer sequences for β-actin (position 103–122 and 642–619) and IL-6 (position 113–132 and 302–283) were taken from Yamamura *et al* [20] and crossed intron-exon boundaries.

Immunoprecipitation and Western Blot Analysis For immunoprecipitation, 2×10^6 stimulated and unstimulated cells were solubilized in a lysis buffer containing 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodiumdeoxycholate, 2% NP-40, 0.2% SDS, 1 mM PMSF, and 50 μg/ml aprotinin. Cell lysates were incubated for 2 h with sepharose CL-4B (Sigma) to which polyclonal goat anti-human IL-6 (British Biotechnology) had been coupled. To elute sepharose-bound protein, pellets were boiled for 5 min in 1X Laemmli buffer [21]. For Western blotting, either eluted protein from cell lysates or acetone precipitated culture supernatants of HMC-1 or KU812 cells and of fibroblasts which had been resuspended in 1X Laemmli buffer were electrophoresed through a sodium dodecylsulfate–polyacrylamide gel electrophoresis on 12.5% mini-gels (Bio-Rad, Richmond, CA) and transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) according to the method of Towbin [22]. Blotted IL-6 was visualized by means of polyclonal goat anti-human IL-6 antibody (British Biotechnology), peroxidase-conjugated rabbit anti-goat IgG $F(ab)_2$ fragment (Dianova, Hamburg, Germany), and a chemiluminescence detection system (ECL; Amersham, Braunschweig, Germany), according to the supplier's recommendations. Blots were exposed to Kodak XAR-5 films. Human rIL-6 which served as positive control was a non-glycosylated *Escherichia coli*–derived protein of 20.66 and 18.8 kDa molecular mass (Hiss Diagnostics). For specificity control, the anti-human IL-6 antibody was preincubated with excess rIL-6 or was replaced by nonimmune goat IgG (Dianova).

Immunoelectron Microscopy Fresh skin specimens (4-mm punch biopsies from foreskin) were preincubated with 1 μg/ml human IgE (Calbiochem, La Jolla, CA) in serum-free Iscove's medium overnight and stimulated with 1 μg/ml rabbit anti-human IgE (Behring, Marburg, Germany) for 16 h. For control, skin biopsies were incubated in medium alone or in medium containing either IgE or anti-IgE. After the incubations, all specimens were processed for immunoelectron microscopy as previously described [23]. Tissue sections were incubated with a polyclonal goat anti-human IL-6 antibody (British Biotechnology) and a peroxidase-conjugated rabbit anti-goat IgG $F(ab)_2$ fragment (Dianova), or with equal amounts of nonimmune goat IgG (Dianova) for specificity control. A mouse anti-human tryptase antibody (AA1) served as positive control for the staining of mast cell granules.

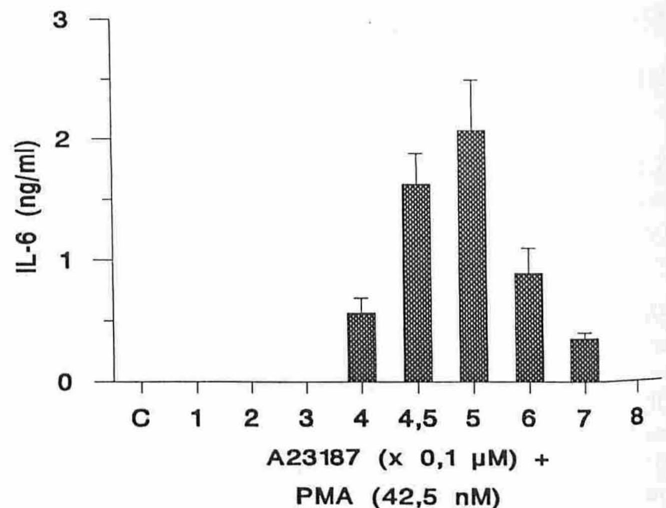

Figure 1. Stimulation of HMC-1 cells with PMA and A23187 induces IL-6 release. HMC-1 cells were treated with *PMA* (42.5 nM) in combination with various concentrations of *A23187* (0.1 to 0.8 μM) for 24 h, and *IL-6* release was measured by ELISA. Untreated cells (*C*, control) and cells that had been treated with either of the stimuli alone (not shown) did not secrete IL-6. *Error bars*, SD of data from three independent experiments (total n = 6).

RESULTS

ELISA In initial concentration dependence studies, we examined the production of IL-6 by HMC-1 and KU812 cells after 24-h treatment with different concentrations of PMA (21.25 nM to 0.34 μM) and/or calcium ionophore A23187 (1 μM to 0.01 μM). HMC-1 cells treated with PMA (42.5 nM) in the presence of various concentrations of A23187 (0.1 to 0.8 μM) secreted substantial amounts of IL-6 in a concentration-dependent fashion **(Fig 1)**, whereas neither agent alone induced significant production of IL-6 in any of the concentrations tested (not shown). Maximal IL-6 release (up to 2.4 ng/ml) was obtained by combined treatment of HMC-1 cells with PMA (42.5 nM) and A23187 (0.5 μM) **(Fig 1)**. KU812 cells, in contrast, produced up to 4.1 ng/ml IL-6 when stimulated with A23187 (0.5 μM) alone, and up to 7-fold more IL-6 (15 ng/ml) compared with HMC-1 cells after costimulation with PMA (42.5 nM) **(Fig 2)**. These optimal doses of PMA and A23187 were used throughout the subsequent experiments. In time course experiments, the release of IL-6 by PMA/A23187-treated HMC-1 **(Fig 3)** and KU812 cells (not shown) started within 4 to 8 h after stimulation, peaked at 48 to 72 h, and declined thereafter.

Bioassay Maximal proliferative response of TEPC 1033 cells, equivalent to 400 pg/ml rIL-6, was achieved using 1:8 diluted supernatants of HMC-1 cells stimulated with PMA (42.5 nM) and A23187 (0.5 μM) for 24 h, suggesting a production of 3200 pg/ml IL-6 bioactivity. In contrast, no IL-6 activity was observed in supernatants from unstimulated cells or from cells exposed to PMA (42.5 nM) or A23187 (0.5 μM) alone. Preincubation of cell culture supernatants with a neutralizing anti–IL-6 antibody (1 μg/ml) completely blocked the proliferative response of the TEPC 1033 cells. In concentrations ranging from 10 to 0.01 μg/ml, the antibody completely inhibited activities of up to 500 pg/ml rIL-6 (not shown).

RT-PCR Analysis In order to determine the time-dependent expression of IL-6 mRNA, HMC-1 cells were stimulated with PMA/A23187 for various times (as indicated), and total cellular RNA was extracted, reversely transcribed into cDNA, and analyzed by semiquantitative RT-PCR analysis. To provide a meaningful comparison of the IL-6 mRNA content of different samples, cDNAs were first normalized to equal concentrations of β-actin

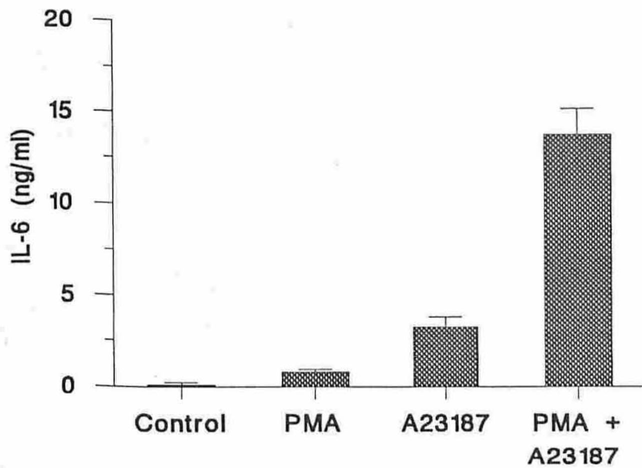

Figure 2. Stimulation of KU812 cells with PMA and/or A23187 induces IL-6 release. Cells were treated with *PMA* (42.5 nM), *A23187* (0.5 μM), or both for 24 h, and *IL-6* secretion was measured by ELISA in comparison with untreated controls (*Control*). *Error bars*, SD of three independent experiments (total n = 6).

PCR product by competitive PCR. Each cDNA was amplified in 10-fold **(Fig 4*a*)** and subsequently in 2-fold dilution steps **(Fig 4*b*)** in the presence of a fixed amount of β-actin control fragment in order to determine the dilution of cDNA required to achieve equal band intensities for both β-actin PCR products. The equalized cDNAs **(Fig 4*c*)** were analyzed for IL-6 transcripts using IL-6-specific primers. In accordance with the ELISA data, IL-6 mRNA was not present in unstimulated cells and was first detectable 2 h after stimulation with PMA/A23187 **(Fig 4*d*)**. Strong and prolonged expression of IL-6 mRNA was observed between 4 and 12 h after stimulation and declined thereafter.

Immunoblot On immunoblot studies, several IL-6 forms could be detected in culture supernatants of PMA/A23187-stimulated HMC-1 and KU812 cells. As illustrated in **Fig 5**, both cell lines secreted four forms of IL-6 of approximately 21, 23, 28, and 30 kDa. All four IL-6 bands could be partially displaced on the blot by preincubation of the anti–IL-6 antibody with an excess of rIL-6 **(Fig 5,** *lanes 8–10*). None of these IL-6 bands was present in the supernatant of unstimulated HMC-1 or KU812 cells. In comparison, IL-1β–stimulated fibroblasts produced two forms of IL-6 protein of about 23 and 30 kDa **(Fig 5,** *lane 3*).

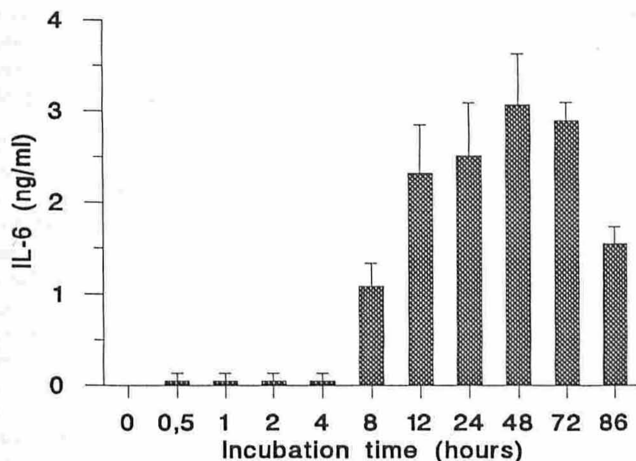

Figure 3. PMA/A23187-costimulated HMC-1 cells time-dependently release IL-6. HMC-1 cells were treated with the combination of PMA (42.5 nM) and A23187 (0.5 μM) for 0.5 to 86 h and *IL-6* secretion was measured by ELISA. *Error bars*, SD of three independent experiments (total n = 6).

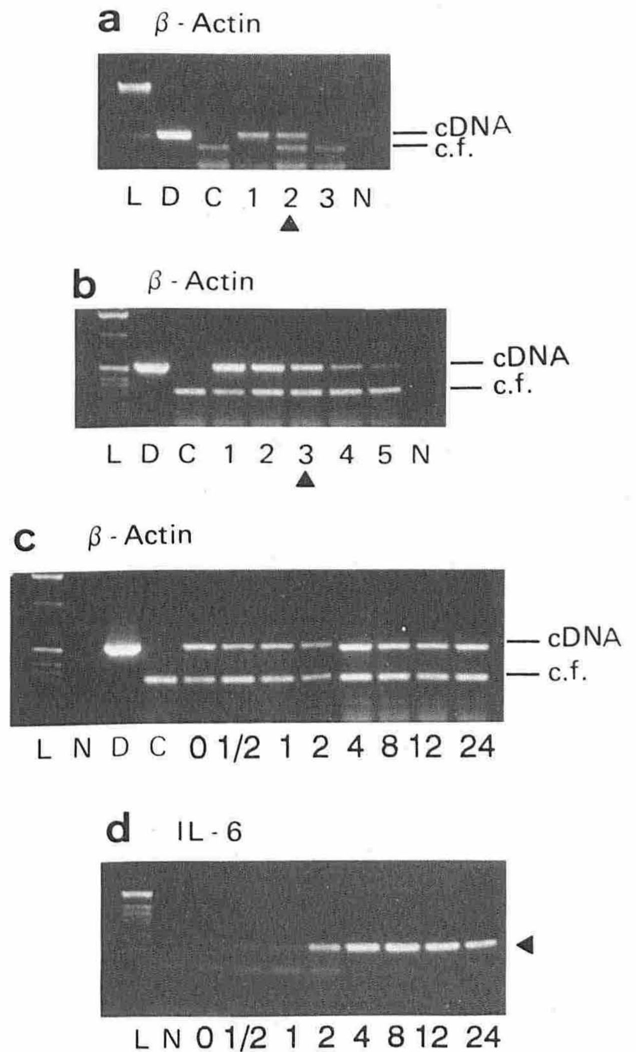

Figure 4. Expression of IL-6 mRNA after costimulation of HMC-1 cells with PMA and A23187 increases with time. HMC-1 cells treated with the combination of PMA (42.5 nM) and A23187 (0.5 μM) for 0.5 to 24 h were analyzed for their *IL-6* mRNA content by semiquantitative RT-PCR. cDNAs of all samples were first equalized for their β-actin concentration by 10-fold (*a*) and 2-fold (*b*) dilution steps of the cDNAs in the presence of a fixed amount of control fragment to obtain equal band intensities of the cDNAs and the control fragment (*arrow*) at each time point analyzed (35 PCR cycles [*c*]). The equalized cDNAs were analyzed for IL-6 content after 36 PCR cycles (*d*). PCR products were resolved on a 1.5% agarose gel and stained by ethidium bromide. *a*) *Lanes 1–3*, cDNA dilutions of 1:10, 1:100, 1:1000 (unstimulated HMC-1 cells) *b*) *Lanes 1–5*: cDNA dilutions of 1:12.5, 1:25, 1:50, 1:100, 1:200 (unstimulated HMC-1 cells); *c,d*), *Lanes 1–8*: cDNAs from HMC-1 cells stimulated with PMA and A23187 for various times (as indicated [h]). To control for contaminations, the PCR reaction mixture was amplified with water alone (*N*), with cDNA alone (*D*), or with control fragment alone (*C*); *L*, 1-kb DNA ladder; c.f., control fragment.

In order to study the expression of intracellular IL-6 protein, we performed immunoprecipitation experiments from lysates of both cell lines followed by immunoblotting of the eluted proteins. Lysates of PMA/A23187-stimulated HMC-1 (not shown) and KU812 cells contained the same four IL-6 isoforms as the respective culture supernatants ranging from 21 to 30 kDa **(Fig 6)**. Lysates of unstimulated cells of both lines, in contrast, did not contain detectable IL-6 protein **(Fig 6)**. The human rIL-6 had a molecular weight of about 21 and 19 kDa **(Fig 5,** *lanes 1, 7,* **Fig 6,** *lane 4*).

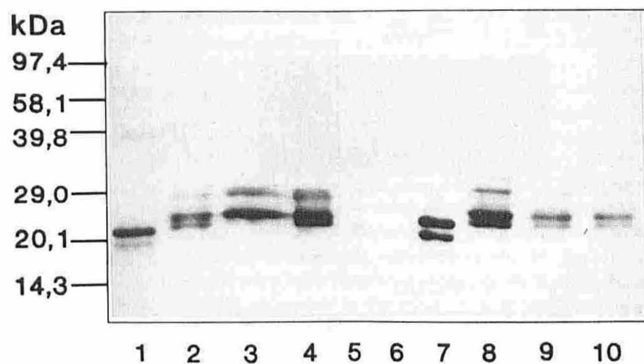

Figure 5. Stimulation with PMA and A23187 induces the expression of 21, 23, 28, and 30 kDa IL-6 in HMC-1 and KU812 cells. Culture supernatants of HMC-1 and KU812 cells that had been incubated for 24 h in the presence (*lanes 2,4*, respectively) or absence (*lanes 5,6*, respectively) of PMA (42.5 nM) and A23187 (0.5 μM) and supernatant of IL-1β (1 ng/ml)–stimulated human fibroblasts (*lane 3*) were analyzed by immunoblotting, as described in *Materials and Methods*. All four KU812 cell-derived IL-6 isoforms could be partially displaced from the blot (*lane 8*) by preincubation of the anti–IL-6 antibody with 350 ng (*lane 9*) or 700 ng (*lane 10*) of human rIL-6; *lanes 1, 7*, human rIL-6.

Immunoelectron Microscopy In order to further examine the biological relevance of our findings in cell lines, we studied the IL-6 expression of human skin mast cells by the use of immunoelectron microscopy. Staining for IL-6 was found in mast cells of skin biopsies preincubated with IgE and stimulated with anti-IgE for 16 h. The electron-dense peroxidase deposits were mainly observed in the intra- **(Fig 7*a*)** and extracellular granules **(Fig 7*b*)**. By contrast, mast cells of unstimulated skin **(Fig 7*c*)** and of skin stimulated with IgE or anti-IgE alone (not shown) did not show labeling. Treatment of skin biopsies with PMA and A23187 induced also appearance of immunoreactive IL-6 in mast cell granules (not shown).

DISCUSSION

The present data demonstrate the ability of human skin mast cells to produce IL-6 after IgE-dependent stimulation and of a mast cell and a basophilic cell line to produce several isoforms of IL-6 after appropriate stimulation. They confirm previous data on IL-6 production in rodent mast cells and extend the spectrum of cytokines reported before for human skin mast cells (IL-4, IL-8, tumor necrosis factor–α) [23–25]. The ability of normal human basophils to produce IL-6 has not been reported before.

Since detailed studies of IL-6 production by cutaneous mast cells and by blood basophils are hampered by difficulties associated with the purification of these cells and their low yield after such procedures, we used the transformed, immature HMC-1 and KU812 cell lines instead for investigation. We showed that both cell types lack expression of IL-6 protein and mRNA in the unstimulated state, in agreement with the immunoelectron-microscopic findings in mast cells of unstimulated skin. They differ, however, from findings in the nasal mucosa where mast cells were found to stain for IL-6 [26]. This discrepancy may be explained by preactivation of mast cells in nasal mucosa due to prior exposure to exogenous allergens or to possible heterogeneity of mast cells regarding not only their protease contents [27], but also their pattern of cytokine production [23,26].

After stimulation with the combination of PMA and A23187, IL-6 mRNA levels were rapidly upregulated and maintained over many hours in HMC-1 cells. This time course of IL-6 mRNA expression is similar to that reported for IL-1-stimulated human

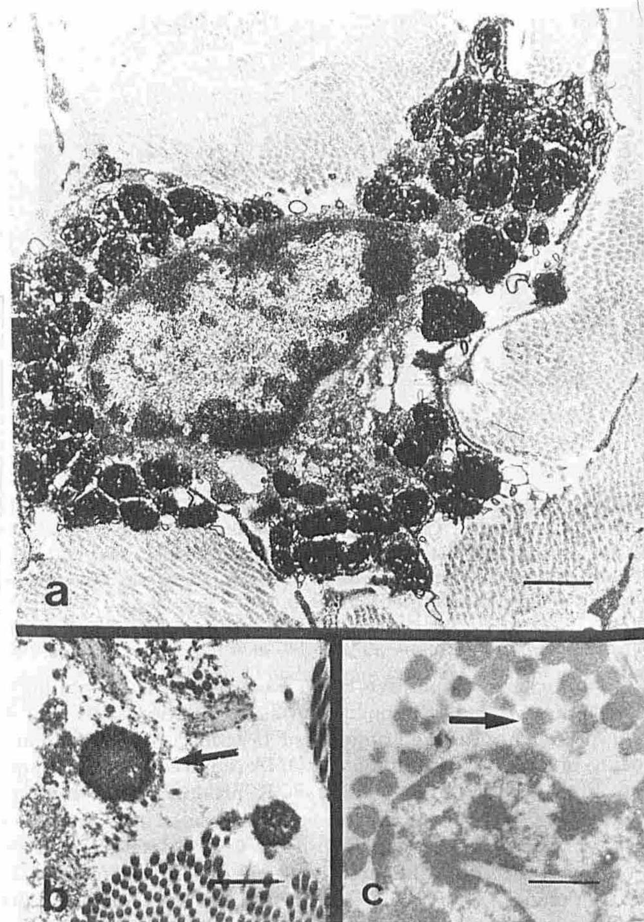

Figure 7. Immunoelectron microscopic analysis of IL-6 expression in human skin mast cells. Skin samples were preincubated with IgE (1 μg/ml) overnight, stimulated with anti-IgE (1 μg/ml) for 16 h, processed for immunoelectron microscopy, and stained for IL-6, as described in *Materials and Methods*. In IgE/anti-IgE-stimulated skin samples, there was strong staining for IL-6 in cytoplasmatic mast cell granules (*a*), and also in extruded extracellular mast cell granules (*arrowhead*) (*b*). Mast cells of skin samples incubated with medium alone (*c*), or with IgE or anti-IgE alone (not shown) did not stain for IL-6. *Scale bars*, 0.1 μm.

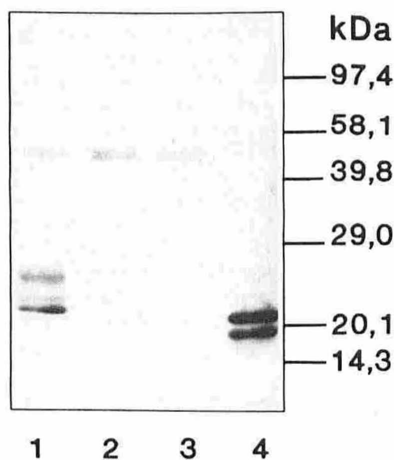

Figure 6. Immunoprecipitation of 21, 23, 28, and 30 kDa IL-6 from homogenates of KU812 cells stimulated with PMA and A23187. Cell lysates were obtained from KU812 cells incubated for 4 h in the presence (*lanes 1,3*) or absence (*lane 2*) of PMA (42.5 nM) and A23187 (0.5 μM) and immunoprecipitated as described in *Materials and Methods* with an anti-IL-6 antibody (*lanes 1,2*) or with non-immune goat IgG (*lane 3*). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting, as described in *Materials and Methods*. *Lane 4*, human rIL-6. The band at about 58 kDa was nonspecific.

synoviocytes [28], but is slow compared with the rapid increase (1 h) and decline (4 h) in IgE-stimulated murine mast cells [29]. The kinetics of IL-8 mRNA production by HMC-1 cells have been shown by us to be on the other hand distinctly faster than that of IL-6, using the same stimuli [23]. This underlines the species-, cytokine-, and possibly also stimulus-dependent differences in the kinetics of cytokine synthesis by these cells.

With respect to secretion of IL-6, the combination of PMA and A23187 induced maximal IL-6 release in the human mast and the basophilic cell line. Moreover, lysates of unstimulated cells of both lines did not contain measurable IL-6, and stimulation of the cells in the presence of cycloheximide completely abolished production of IL-6 protein (not shown), indicating *de novo* generation of IL-6. These data suggest that the efficient induction of IL-6 in both cell lines requires delivery of at least two signals, one generated by an activation of the protein kinase C and the other by elevation of $[Ca^{2+}]_i$, as has been suggested for FcεRI-dependent stimulation of cytokine production in mast cells [6,30]. The immunoblot data show furthermore that both human mast and basophilic cells produce and secrete several IL-6 isoforms after stimulation. In accordance with other types of cells such as monocytes/macrophages, fibroblasts, and endothelial cells where at least three O-glycosylated IL-6 isoforms of 23 to 25 kDa and three O- and N-glycosylated species of 27 to 30 kDa could be discriminated [31–33].

The rapid synthesis and release of substantial amounts of IL-6 within a few hours after stimulation from human mast and basophilic cell lines, as reported here, could be of particular importance in the induction of inflammatory responses. Its significance in the course of diseases in which these cells may play a role [3,4,8–11] has yet to be established, although our observations emphasize the potential pathophysiological significance of mast cell– and basophil-derived cytokines for persistent IgE-dependent as well as nonspecific inflammatory responses.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Mo 462/2-2). The authors are grateful to Mrs K. Köster and Mrs V. Odenwald for excellent technical assistance; to Prof. T. Diamantstein, Department of Immunology, Berlin, for helpful discussion; to Dr. Butterfield, Mayo Clinic, for providing the HMC-1 cells; to Drs. Rietschel, Borstel, Germany, and Valent, Vienna, Austria, for the KU812 cells; and to Dr. S. Wall, Southampton, UK, for the AA1 antibody.

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