

Expression of Actin and Tubulins in Purified Human Basophil Leukocytes upon Stimulation with IL-3

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Key Words

Basophils · IL-3 · Actin · Tubulins

Abstract

Background: Pharmacological and morphological results indicate that the cytoskeleton proteins tubulins and actin play a role in the histamine release process in basophil leukocytes and mast cells. In this report, we investigate the expression of these cytoskeleton proteins in purified human basophils upon stimulation with anti-IgE and IL-3. **Methods:** Human basophils were purified using a negative selection procedure. They were incubated for 6 h with anti-IgE and/or IL-3 in radiolabeling media. Proteins in the cells were analyzed by two-dimensional gel electrophoresis and autoradiography. Actin and α - and β -tubulin were identified by amino acid sequence analysis or matrix assisted laser desorption/ionisation time-of-flight mass spectrometry. The synthesis of these proteins under different experimental conditions was evaluated by densitometry. **Results:** Actin is regulated dose dependently by IL-3 with an optimum concentration of 100 ng/ml, corresponding to the optimal IL-3 concentration for enhancement of histamine release. The synthesis of tubulins was not significantly upregulated by IL-3 or anti-IgE alone, but there was a slight and significant upregulation of tubulins upon stimulation with both IL-3 and anti-IgE in doses optimal for maximal histamine release. **Conclusions:** The de novo synthesis of actin is

regulated dose dependently by IL-3 in purified human basophils in short-term culture. The optimum IL-3 concentration is the same as for enhancement of histamine release, suggesting that the same processes that regulate IL-3-induced enhancement of histamine release also regulate the expression of actin. The finding that the expression of the tubulins is not upregulated by IL-3 alone suggests that regulation of tubulin synthesis is not a pathway by which IL-3 in itself primes basophil histamine release.

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Introduction

Basophil leukocytes are involved in the late-phase reaction of allergy, as they invade the inflamed tissue hours after the initial allergic reaction [1, 2]. Ample evidence exists that the cytoskeleton proteins tubulins and actin play a role in the process of histamine release from basophil leukocytes. Thus, morphological data show that granules in mast cells are connected to a mesh of microfilaments that follows the granules during exocytosis [3]. The kinetics of filament actin (F-actin) polymerization and histamine release with IgE-dependent and -independent stimulants are identical [4], and incubation with the microfilament-disrupting agents cytochalasins modulates histamine release from mast cells [5–7]. Also, some results indicate that actin plays a role in the coupling between the IgE-Fc ϵ RI complex and downstream signaling pathways [8].

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The tubulin-depolymerizing drugs colchicine and vinblastine inhibit histamine release from basophils [6, 9]. It has been reported that the uptake of Ca^{2+} into cells upon stimulation with compound 40/80 was inhibited by colchicine and vinblastine. Also, the Ca^{2+} release from intracellular stores was inhibited by these agents [10, 11].

In order to identify anti-IgE- and IL-3-sensitive proteins in human basophils, we used two-dimensional gel electrophoresis (2-D-gels) to separate and analyze the expression of proteins from purified human basophils. Using these data, we found that actin is very sensitive to IL-3, while the tubulins did not show significant regulation by IL-3 and only a modest though significant increase in de novo synthesis upon stimulation with IL-3 and anti-IgE together.

Materials and Methods

Materials

Dyna-Beads were from Dynal (Oslo, Norway). The monoclonal antihuman antibodies CD3, CD4, CD8, CD14, CD16, CD19 and monoclonal anti-IgE were from Dakopatts (Glostrup, Denmark). Polyclonal goat antihuman anti-IgE was from Behringwerke (Marburg, Germany). Recombinant human (rh)IL-3 was obtained from Genzyme (USA). ^{35}S -Cys/Met (PromixTM) was supplied by Amersham (UK). Percoll, Ficoll-Hypaque and Ampholines pH 8–10, pH 7–9 and pH 3.5–10 were acquired from Pharmacia Biotech (Uppsala, Sweden), and Ampholines pH 5–7 from Serva (Heidelberg, Germany). Films for autoradiography were Kodak X-omatic. PVDF membranes were from MSI (Westboro, Mass., USA). The KU812 cells were supplied by Dr. Lars K. Poulsen (Allergy Unit, National University Hospital, Copenhagen, Denmark). All other reagents used were of analytic grade.

Purification of the Basophil Leukocytes

The basophil leukocytes were purified using Dyna-Beads in a negative or positive selection procedure. The negative procedure was performed according to the methodology described previously [12]. Blood (50 ml) was obtained by venipuncture from healthy volunteers. The blood was layered on Percoll gradients and centrifuged at 400 g for 20 min at room temperature. The rest of the procedures were performed at 4°C. The interphase was harvested and washed and the basophil leukocytes were counted. Contaminating non-basophil cells were removed by negative selection using Dyna-Beads (CD8, CD19, CD4, CD 14, CD16, CD3). After negative selection, the cells were counted and washed in RPMI-1640 without serum. Only samples with more than 89% basophil leukocytes were used for experiments. The average yield of these samples was 1.3×10^6 basophils.

For positive selection, the cells in the interphase were incubated with Dyna-Beads coated with mouse monoclonal anti-human IgE antibodies (ratio of basophils to Dyna-Beads 1:4) for 1 h with gentle rolling. Dyna-Beads with bound basophil leukocytes were separated using a magnet. The purity of these samples was above 96%, with a total average yield of 0.9×10^6 basophils.

KU812 cells were grown in RPMI-1640 (10% HSA). They were washed in serum-free RPMI-1640 before application on the gels.

Histamine Measurement

Histamine was measured in the pellet and the supernatant according to the methodology previously published [13]. Histamine release was expressed as a percentage of the total content of histamine. All measurements were made in triplicate.

Purification of Mononuclear Cells

For purification of mononuclear cells, blood was layered and centrifuged on Ficoll-Hypaque according to the instructions of the manufacturer.

2-D-Gels

The cell pellet was dissolved in lysis buffer (9.8 M urea, 2% (w/v) NP40, 2% Ampholine pH 7–9, 100 mM dithiothreitol) and applied to 2-D-gels. This was performed as described by Celis et al. [14], except that we used the Protean xi 2-D Cell from Bio-Rad. Molecular weight and pI were assigned according to comigration with 2-D-gel protein standards.

For amino acid sequence analysis, proteins in the 2-D-gel were transferred to PVDF membranes in a Tris (50 mM)-boric acid (50 mM) buffer at 130 mA overnight.

Autoradiography

Gels were fixed in 50% methanol and 10% acetic acid for 4 h and washed several times in 30% ethanol. Subsequently, they were dried in a Bio-Rad gel dryer and exposed to X-ray film in a cassette at room temperature. The exposure time varied from 5 to 14 days.

Amino Acid Sequencing

The proteins in the gels were transferred to PVDF membranes as described above and stained with amido black (0.1% amido black, 45% MeOH, 7% acetic acid). Before the sequence analysis was carried out, the membrane piece was washed twice in 50% methanol including a few minutes of sonication during the first wash. Then the membrane piece was transferred to the blot cartridge of the 'Proclise' protein sequencer (model 494A, Perkin-Elmer ABD, Foster City, Calif., USA) and analyzed using essentially the preprogrammed 'Blot' cycles. All solvents and reagents were from Perkin-Elmer ABD, except methanol and solvent B for the HPLC separation of the phenylthiohydantoin amino acid derivatives.

Mass Spectrometry

Spots were excised from the gel and digested with trypsin essentially as described by Wilm et al. [15]. In short, the gel piece was cut into small cubes and washed for 1 h in 100 mM NH_4HCO_3 (AB), and excess liquid was removed. Then the proteins were reduced for 30 min at 60°C in 100 µl of 4 mM dithiothreitol in 100 mM AB, cooled and alkylated by addition of 10 µl of 100 mM iodoacetamide followed by 30 min of incubation at room temperature in the dark. Excess liquid was removed and the gel pieces were washed for 1 h in 50% acetonitrile in 100 mM AB followed by shrinkage in acetonitrile and vacuum centrifugation. The tube with the dry gel pieces was placed in an ice bath and allowed to swell in 25 mM AB including 2 µg/100 µl of modified trypsin (Promega). After 45 min, the remaining supernatant was removed and buffer added to cover the gel pieces during overnight incubation at 37°C. The next day, the supernatant was removed and combined with 2 consecutive extractions with 50 µl of 0.1% trifluoroacetic acid in 60% acetonitrile. The extract was dried in a vacuum centrifuge to near dryness followed by addition of 5 µl of 0.1% trifluoroacetic acid in 30% acetonitrile. The extracted

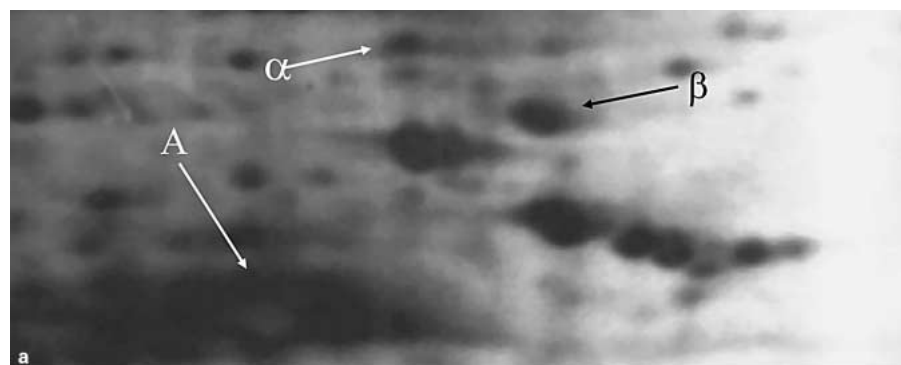
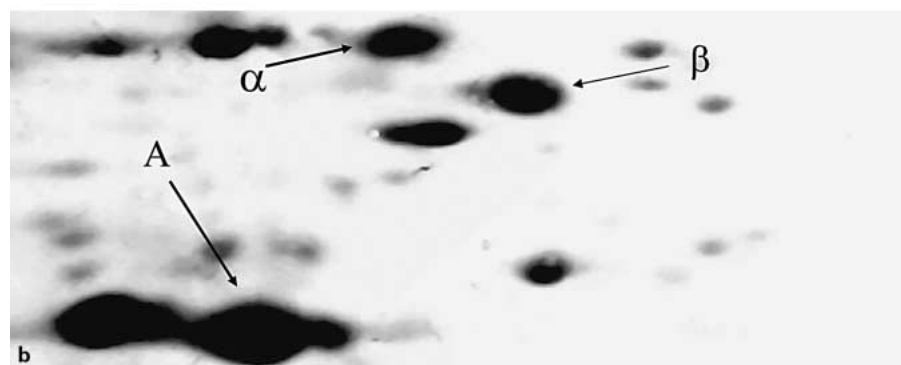


Fig. 1. Identification of actin and α - and β -tubulin on 2-D-gels of human basophil proteins. Parallel samples of radiolabeled proteins from purified human basophils and proteins from KU812 cells (a basophil-like cell line) were applied to 2-D-gels. **a** Autoradiography of the gel with radiolabeled human basophil proteins. **b** Amido black-stained PVDF blot of the 2-D-gel with KU812 cell proteins. The proteins were identified by amino acid sequence analysis and mass spectrometry as described in the text. α = α -Tubulin; β = β -tubulin; A = actin; MW = molecular weight (kD); pI = isoelectric point.



peptides were analyzed in a matrix assisted laser desorption/ionization time-of-flight mass spectrometer with reflector and delayed extraction option (Biflex, Bruker-Franzen, Bremen, Germany). For analysis, 0.4 μ l of the sample was mixed with 0.1 μ l of internal standard mixture (angiotensin II and dynorphin, 0.06 and 1 pmol/ μ l, respectively) and 0.5 μ l of matrix solution (α -cyano-4 hydroxycinnamic acid in acetonitrile-methanol, Hewlett Packard). Then, 0.5 μ l of the mixture was applied to the probe and allowed to dry before introduction into the mass spectrometer. The peptide molecular mass fingerprint was used to search the SWISS-PROT database using the 'PeptIdent' tool on the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics [16].

Measurement of the Expression of Actin and Tubulins in Basophils

The expression of proteins in human basophils upon stimulation with rhIL-3 and/or anti-IgE was measured against controls from a database of 2-D-gels of proteins from purified human basophils [17]. Human basophils were purified and shortly after used for experiments. The cells were incubated at a concentration of 5×10^6 /ml in serum-free RPMI-1640 deficient of methionine, cysteine and cystine with 0.3 mCi/100 μ l ^{35} S-Met/Cys (specific activity 1,000 Ci/mM). One stimulation experiment consisted of paired samples subjected to different stimulations. The total number of cells in an experiment varied between 0.7×10^6 and 1.8×10^6 . Within an experiment, each sample consisted of equal amounts of basophil leukocytes. Anti-IgE and rhIL-3 were added as indicated. The cells were incubated for 6 h at 37 °C and 5% CO₂. Viability of the cells was assessed after incubation with trypan blue exclusion and consistently exceeded 95%. After incubation, the cells were pelleted (400 g, 4 min), dissolved in lysis

buffer and applied to the two-dimensional gels. The amount of anti-IgE used was the optimal dose for histamine secretion from the donors as determined by earlier experiments. Buffer was added as controls. Parallel samples were run for the histamine measurements.

Gels were analyzed both visually and using a tabletop scanner (Mustek MFS-6000CX). The proteins were quantified using a 2-D-gel densitometry software program from Phoretix (UK). The spot volume was defined as the summation of the value of the intensities of each pixel in the spot. To correct for differences in applied material to the gel, the spot volumes were expressed in relation to the total amount of protein on the gel (defined as the summation of the value of the intensities of each pixel in the gel). Thus, the results of the densitometric measurements are presented as follows: spot percentage of total gel volume = (spot volume)/(total gel volume) $\times$ 100.

Statistics

Data were analyzed using Wilcoxon's signed rank test for matched pairs. The data on the dose-response relationship between IL-3 concentration and actin expression were analyzed using an ANOVA on ranks (Friedman).

Results

Identification of Actin and Tubulins in Basophils

Parallel samples of radiolabeled proteins from purified human basophils and proteins from KU812 cells (a basophil-like cell line) were applied to 2-D-gels. The gel with

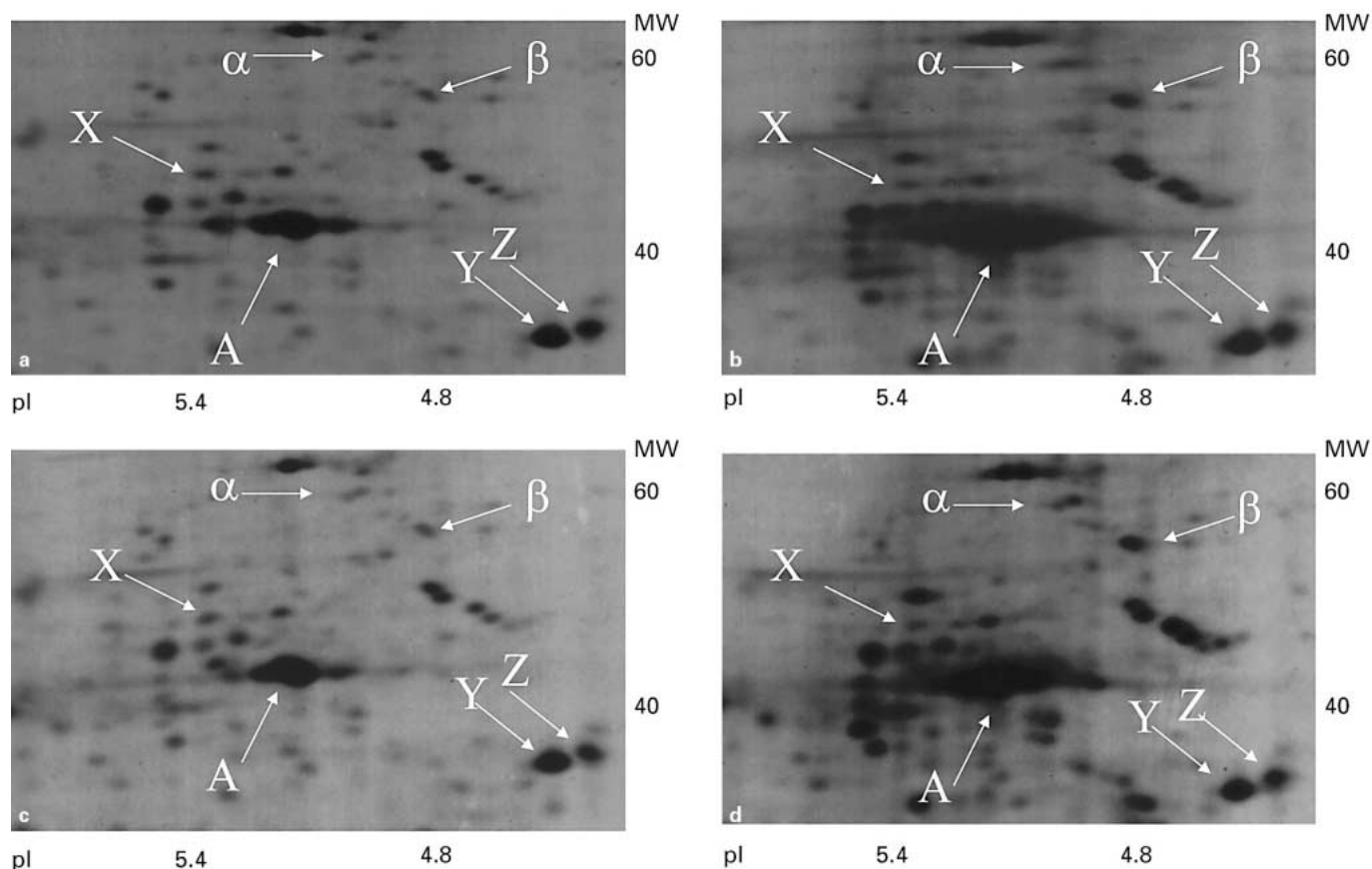


Fig. 2. Gels from one experiment in which purified human basophils were incubated with radiolabeling media and buffer (control) (**a**), rhIL-3 100 ng/ml (**b**), anti-IgE 50 IU/ml (**c**) or anti-IgE 50 IU/ml + rhIL-3 100 ng/ml (**d**) for 6 h. Cells were lysed, and the proteins were separated on 2-D-gels and visualized by autoradiography. Thus, the spots represent proteins synthesized during the incubation period. The arrows point to actin (A), α -tubulin (α) and β -tubulin (β). The proteins marked X, Y and Z are examples of proteins with uniform expression under the different experimental conditions. MW = Molecular weight (kD); pI = isoelectric point.

radiolabeled human basophil proteins was subjected to radiography (fig. 1a). The gel with KU812 cells was transferred to PVDF membrane and stained with amido black (fig. 1b). α - and β -tubulin were identified on the KU812 gel by N-terminal amino acid sequence analysis. α - and β -tubulin were identified on the gels of human basophil proteins by having the same migratory pattern as α - and β -tubulin in KU812 cells. Actin was identified by mass spectrometry of the protein excised directly from the gel of human basophil proteins.

Synthesis of Actin Is Enhanced by rhIL-3

The expression of actin in the basophils upon rhIL-3 and/or anti-IgE treatment of the cells was measured as described in Materials and Methods. Figures 2a–d are a representative sequence of autoradiographs of gels from one donor, showing the synthesis of actin in basophils

upon stimulation with anti-IgE, rhIL-3 or both. Clearly, the expression of actin is enhanced by stimulation with rhIL-3. As is obvious from the gels, other proteins are upregulated as well, and one could get the impression that rhIL-3 enhances the synthesis of any protein in the basophils. However, some proteins on the gels showed uniform expression under all experimental conditions, like proteins X, Y and Z.

Figures 3a–c represent densitometric measurements of actin in 11 experiments. There was a significant 2-fold increase (median) in the expression of actin upon stimulation with rhIL-3 compared to controls. There was also a small though significant increase in expression of actin upon incubation with anti-IgE (fig 3b). The purity of the basophil leukocytes in these experiments was $94.1 \pm 0.9\%$ (mean $\pm$ SEM; range 89.0–96.4%).

Fig. 3. a–c The experiment described in figure 1 was repeated 11 times with purified human basophils, and densitometric measurements of actin under the different experimental conditions compared to the controls are shown: with rhIL-3 100 ng/ml (**a**), with anti-IgE 50 IU/ml (**b**) or with rhIL-3 100 ng/ml + anti-IgE 50 IU/ml (**c**). Some gels were missing, therefore only 10 experiments are shown in **a** and **b**. The bars represent median values. **d** Histamine release (HR; mean $\pm$ SEM) in the samples of all 11 experiments. * $p < 0.05$, ** $p < 0.01$ compared to control.

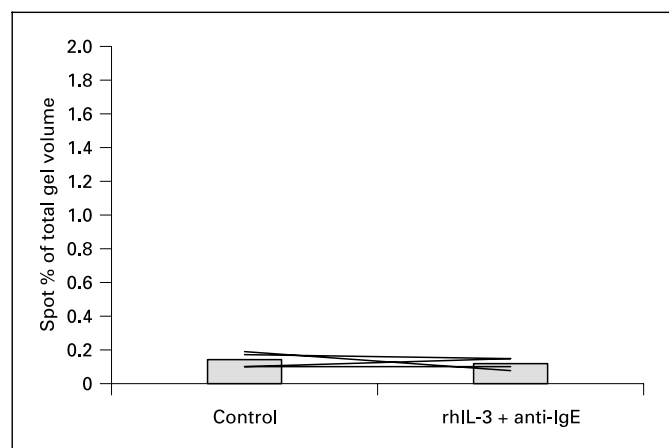
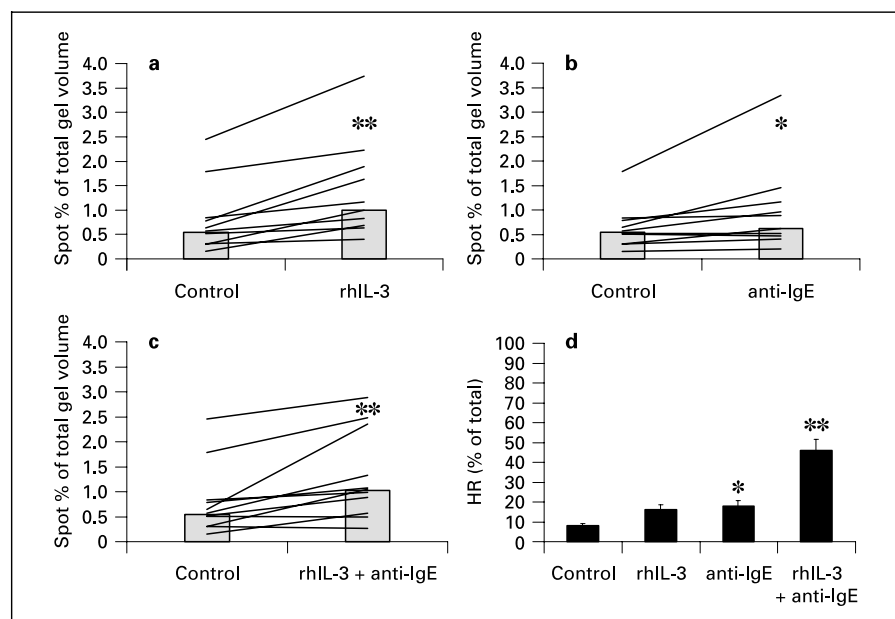


Fig. 4. Control experiments. As the contaminating cells in the basophil preparations were mononuclear cells, parallel experiments were performed with purified mononuclear cells. The fraction of basophils in these samples was below 2%. The diagram shows densitometric measurements ($n = 4$) of actin in mononuclear cells stimulated with anti-IgE (50 IU/ml) and rhIL-3 (100 ng/ml) compared to control cells.

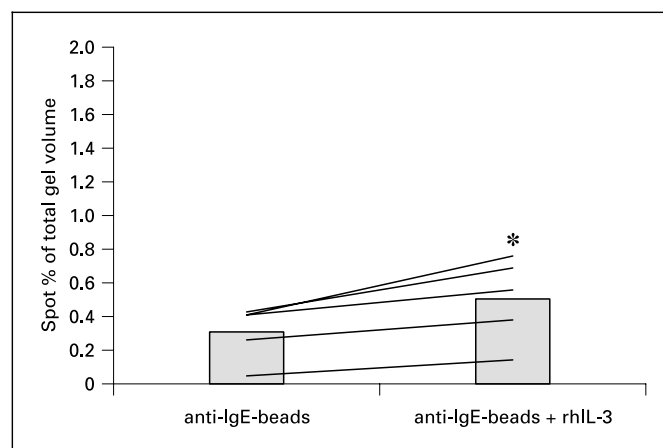


Fig. 5. Control experiments. Basophils were purified using a positive selection procedure with anti-IgE-coated beads. Basophils were incubated with or without 100 ng/ml rhIL-3. The purity of basophils in these experiments was above 96%. After incubation, the cells were processed as described in the legend to figure 2. The figure shows densitometric measurements of autoradiographic spots representing actin in these experiments ($n = 5$). Bars represent medians. * $p < 0.05$.

As the contaminating cells in the purified samples were mainly lymphocytes, control experiments were conducted in which purified mononuclear cells were obtained and stimulated like the basophils. The content of basophil leukocytes in these samples was below 2%. There was no regulatory effect of any stimulation in these experiments (fig. 4).

Further control experiments were performed, in which basophils were purified by a positive selection procedure with Dyna-Beads coupled to anti-IgE. The purity of these samples was $97.6 \pm 0.5\%$ (mean $\pm$ SEM; range 96.0–99.0%). Densitometric measurements (fig. 5) showed a significant increase in the synthesis of actin by a factor of 1.6 ($p < 0.05$) after rhIL-3 stimulation (100 ng/ml).

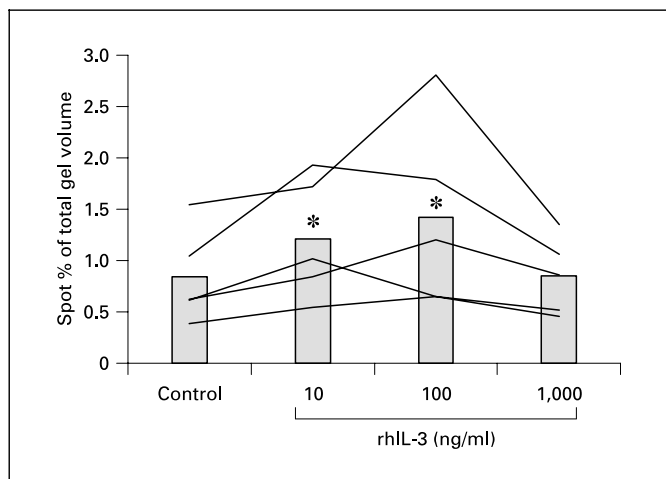


Fig. 6. Densitometric measurements of actin in basophil leukocytes after incubation with different concentrations of rhIL-3 for 6 h (n = 5). The expression of actin was significantly higher with 10 and 100 ng/ml rhIL-3 compared to the control (* p < 0.05).

Five experiments were performed to find a possible dose-response relationship between rhIL-3 and the expression of actin. Previous experiments showed maximal rhIL-3-induced histamine release from purified basophils at a concentration of 100 ng/ml rhIL-3 [18]. As seen in figure 6, we also found maximal enhancement of actin expression at that concentration of rhIL-3.

Synthesis of α - and β -Tubulin upon Incubation with anti-IgE and rhIL-3

In the same gels as were used for measuring actin (fig. 2), the expression of α - and β -tubulin was measured. Some of the results are shown in figure 7. Densitometric measurements of the expression of the proteins showed a nonsignificant enhancement upon stimulation with 100 ng/ml IL-3 (mean percentage of gel volume increased from 0.029 to 0.038% and from 0.040 to 0.048% for α - and β -tubulin, respectively). However, there was a significant increase (p < 0.05) upon combined stimulation with rhIL-3 (100 ng/ml) and anti-IgE (50 IU/ml). In mononuclear cells, rhIL-3 and anti-IgE did not have any influence on the synthesis of α - or β -tubulin (results not shown).

Discussion

In this article, we report that the synthesis of actin is regulated dose dependently by rhIL-3. This conclusion was based on densitometric measurements of autoradio-

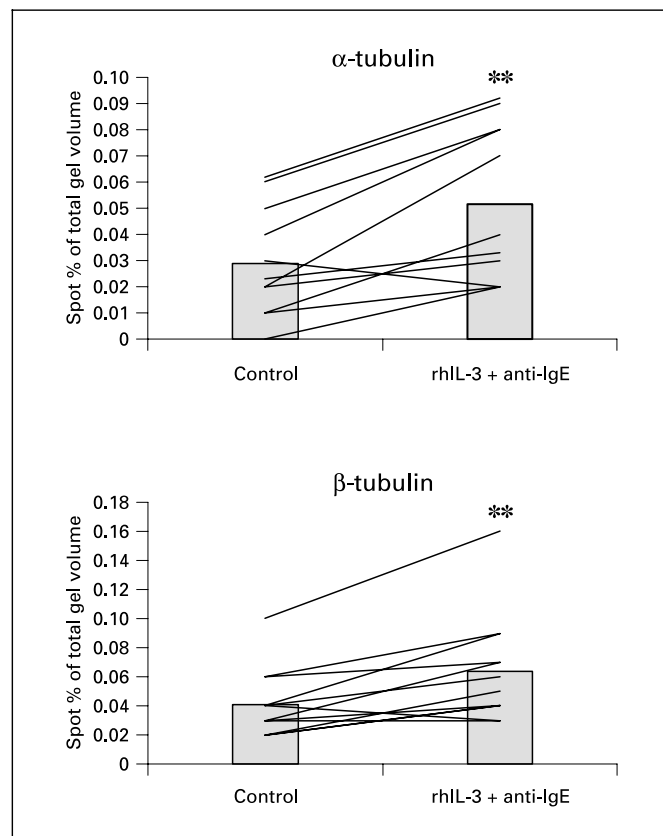


Fig. 7. Densitometric measurements of spots representing α - and β -tubulin in basophils stimulated with anti-IgE (50 IU/ml) and rhIL-3 (100 ng/ml) compared to control cells. ** p < 0.01.

graphs of de novo synthesized actin. IL-3 is known as a basophilopoietin sustaining the viability of basophils. Therefore, one would expect IL-3 to enhance the synthesis of any protein in basophils. However, as reported here, we found several basophil proteins that were not affected by incubation with IL-3. Secondly, we corrected our measurements with the total amount of synthesized proteins so that we can conclude that the relative amount of newly synthesized actin is increased upon incubation with rhIL-3.

IL-3 plays a major role in the regulation of basophil differentiation, survival and secretion. Basophils have receptors for IL-3 on their surface [19, 20]. IL-3 supports intracellular histamine production in human umbilical cord blood mononuclear cells [21] and bone marrow suspension cultures [22]. Based on morphological criteria [23, 24] or cell surface marker profile [22], it has been shown that IL-3 supports differentiation of predominantly basophils but also mast cells from human bone marrow cultures.

IL-3 regulates the survival of basophils; in one experiment in which basophils were cultured without IL-3, only 1% were alive after 14 days, while in the samples with IL-3 added, the basophil viability after 14 days was 45% [25]. Later, it was found that IL-3 inhibits basophil apoptosis [26].

Several groups have reported that IL-3 enhances histamine [27–31], leukotriene [28, 30, 32] and IL-4 [33–36] secretion upon stimulation with different secretagogues.

IL-3 enhances histamine secretion after anti-IgE, formylated methionin-leucin-phenylalanin, A23187 and C5a stimulation, even though these stimulators use different signal transduction mechanisms [29–31, 37]. Therefore, it is thought that IL-3 modulates histamine release at central (proximal) points in the signal transduction mechanism, and several observations support that [38–40]. IL-3 priming makes basophils secrete leukotrienes upon stimulation with C5a [27, 30, 32]. In addition, IL-3 obliterates the inhibitory effect of glucocorticosteroids on histamine secretion by basophils [41].

To our knowledge, this report is the first to demonstrate that rhIL-3 actually increases the synthesis of actin. This is interesting because both morphological and pharmacological observations [6, 7, 9, 42] indicate that actin plays a role in the histamine release process in basophils and mast cells.

It is known that F-actin is formed by polymerization of globular actin in basophils upon stimulation with secretagogues [3]. Interestingly, the kinetics of changes in relative F-actin in cultured human basophils are different upon stimulation with anti-IgE and formylated methionin-leucin-phenylalanin, but parallel the kinetics of histamine release upon stimulation with the same agents [4].

We found that IL-3 modulates the new synthesis of actin with the same optimal concentration as we have found for histamine release under the same (serum-free) experimental conditions, suggesting that the same processes that regulate IL-3-induced enhancement of histamine release also regulate the expression of actin.

Analysis of the synthesis of α - and β -tubulin showed that only the combined stimulation with rhIL-3 and anti-IgE increased the synthesis of tubulins (by a factor of 1.5). It has long been recognized that tubulins play a role in the histamine release process in basophils and mast cells. Tubulin-depolymerizing drugs like colchicine inhibit histamine release, while tubulin-stabilizing agents such as D₂O enhance histamine release [6, 7, 9, 42, 43], indicating that polymerization of tubulin is a part of the process leading to histamine release. We found a similarity between the amount of histamine release and the amount of tubulin expression, thereby providing stoichiometric data suggesting that tubulins do play a role in basophil histamine release. However, the fact that the expression of the tubulins was not upregulated by IL-3 alone suggests that regulation of tubulin synthesis is not a pathway by which IL-3 in itself primes basophil histamine release.

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