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Previous studies have indicated a redundancy in the effects of the cytokines, IL-3, IL-5, and nerve growth factor (NGF) on acute priming of human basophils. In the current study, we have examined the effects of these three cytokines on 18-h priming for leukotriene C4 generation, their ability to induce FcεRIβ mRNA expression, or their ability to sustain basophil viability in culture. We also examine a variety of the signaling steps that accompany activation with these cytokines. In contrast with the ability of IL-3 to alter secretagogue-mediated cytosolic calcium responses following 18-h cultures, 18-h treatment with IL-5 or NGF did not affect C5a-induced leukotriene C4 generation or alter C5a-induced intracellular Ca\(^{2+}\) concentration elevations. IL-3 and IL-5, but not NGF, induced FcεRIβ mRNA expression and all three improved basophil viability in culture with a ranking of IL-3 > IL-5 ≥ NGF. All three cytokines acutely activated the extracellular signal-regulated kinase pathway and the signaling elements that preceded extracellular signal-regulated kinase and cytosolic phospholipase A\(_2\), phosphorylation, consistent with their redundant ability to acutely prime basophils. However, only IL-3 and IL-5 induced Janus kinase 2 and STAT5 phosphorylation. This pattern of signal element activation among the three cytokines most closely matched their ability to induce expression of FcεRIβ mRNA.

Induction of the sustained calcium signaling that follows overnight priming with IL-3 appeared to be related to the strength of the early signals activated by these cytokines but the relevant pathway required was not identified. None of the signaling patterns matched the ability of the cytokines to promote basophil survival.


Stimulation of human basophils elicits the release of preformed mediators (histamine) and de novo-synthesized lipid mediators (leukotriene C4 (LTC4))\(^3\) \((1, 2)\) but the profile of mediators depends on both the particular secretagogue and the cytokine environment. For human basophils, a variety of cytokines (e.g., IL-3, IL-5, GM-CSF, and nerve growth factor (NGF)) enhance mediator secretion, but these cytokines alone are poor stimuli for mediator release (3–7). These cytokines are also found at sites of allergic inflammation (8–14), suggesting that they can modify allergic reactions by priming basophil secretion as well as affecting the function of other leukocytes. Understanding the evolution of the allergic inflammation reaction is confounded by the number of cytokines present and the apparent redundancy in the actions of many cytokines.

For studies of human basophils, we have used as a model of the priming effect induced by IL-3 the ability of IL-3 to enhance LTC4 release following stimulation with C5a. C5a is a strong secretagogue for histamine release but for basophils from most donors does not induce LTC4 release (15–17). Preincubation of the cells with cytokines (IL-3, IL-5, GM-CSF, and NGF), for only 5–15 min, “permits” C5a to induce marked LTC4 release (3, 4, 16, 17). Although each of these cytokines appears to prime the basophil—regardless of LTC4 release—to a similar extent following a short incubation, it is also known that receptor expression for IL-3, IL-5, and GM-CSF, i.e., the receptors with α subunits that share a common β subunit, is markedly different for these three cytokines. This raises the possibility that even between these three cytokines that not all priming effects are equivalent. With respect to NGF, which does not share the same subunit construction as IL-3, IL-5, and GM-CSF, it also seemed likely that priming might be qualitatively distinct beyond the initial acute effects. We hypothesized that this array of cytokines is not strictly redundant in their actions and that it would be possible to distinguish among IL-3, IL-5, and NGF on the basis of the other changes induced in basophils. There are already indications that this is true: IL-3, but not IL-5, is capable of inducing IL-13 secretion. IL-3 synergistically potentiates C5a-induced secretion of IL-13 from human basophils whereas IL-5 is far less effective (18). IL-3, but not IL-5, potentiates Ag-induced, or a combination of Ag- and eosinophil-induced, IL-4 production from these cells (19). In an effort to better understand the significance of the multiple cytokines present during allergic inflammation, the current studies further define some of the distinguishing characteristics of three cytokines, IL-3, IL-5, and NGF, and explore some of the relevant signaling steps that distinguish the activities of these three cytokines.

Materials and Methods

Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, d-glucose, NaF, Na PyO\(_4\), Na\(_2\)VO\(_4\), 2-ME, Nonidet P-40, Tris-HCl (Sigma, St. Louis, MO); crystallized human serum albumin (HSA) (Miles Laboratories, Elkhart, IN); FCS, gentamicin (Life Technologies, Grand Island, NY); RPMI 1640 containing 25 mM HEPES and l-glutamine (BioWhittaker,
Pipes and media

Pipes-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. PAG-EDTA consisted of PAG supplemented with 4 mM EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. ESB is NOVEX electrophoresis sample buffer (NOVEX, San Diego, CA) containing 5% 2-mercaptoethanol (2-ME) and 2% SDS. The sensitivity of the subsequent blotting to the choice of stripping agent determined which of these two were used.

Basophil purification

For most of these experiments, residual cells of normal donors undergoing leukapheresis were enriched in basophils using a combination of Percoll density gradients and countercurrent-flow elutriation, as previously described (20, 21). The cells were further purified by negative selection using a MACS basophil isolation kit (Miltenyi Biotec, Auburn, CA). More recently, we have used a mixture of Abs for negative selection from Stem Cell Technologies (basophil purification kit; Vancouver, British Columbia, Canada) and columns from Miltenyi (negative selection of basophils was determined by Alcian blue staining (22) and from these leukapheresis packs, generally exceeded 99%). For the basophil survival studies, basophils were enriched from fresh blood obtained by venipuncture. In these instances, the cells were first enriched for basophils using a double Percoll step gradient (1.066/1.079 g/ml) and purified to >90% using the Stem Cell reagents and Miltenyi columns as described above.

Basophils collected in PAG if not solely leukapheresis were released histamine and to generate LTC4 in response to a combination of cytokines (IL-3, IL-5, or NGF) and C5a, and IL-3 affects the basophil response directly (3, 4, 17, 23). We have previously confirmed that basophils were the major source of LTC4 release stimulated by IL-3 (15 min or 18 h) or IL-3 or NGF under our experimental conditions (data not shown). Therefore, the basophil-enriched preparations used for the experiments where only histamine LTC4 release and LTC4 generation were assessed had a somewhat lower mean purity (64 ± 17%; range, 47–99%).

Phosphorylation of ERKs and cPLA₂

The phosphorylation of ERKs was assessed using an anti-phospho-ERK Ab and, in some early experiments, the electrophoretic mobility shift using anti-ERK1 and anti-ERK2 Abs as described previously (23–25). The phosphorylation of cPLA2 was assessed from the change in its electrophoretic mobility as detected by anti-cPLA2 Ab (22, 26, 27).

Immunoprecipitation

After stimulating (cytokines or anti-IgE) basophils (1.5–5 × 10⁶ cells/sample) in PAGCM buffer at 37°C, reactions were stopped by adding ice-cold PAG, or directly centrifuging for 5–10 s in an Eppendorf microtube set for 16,000 × g. The cell pellets were immediately lysed in CLB. Lysates were precleared with protein G-Sepharose beads for 1 h at 4°C to remove any nonspecific binding to the beads. The lysates were then incubated with 1 µg/ml specific Ab to prebound protein G-Sepharose beads at 4°C. After a 1-h incubation, the beads were washed three times with CLB buffer. The immunoprecipitated proteins were eluted by boiling in ESB. Electrophoresis, transfer, and immunoblotting with anti-phospho-ERK Ab (4G10) was performed as described above. The Ab was stripped from the membranes, and then membranes were reprobed with the indicated Abs.

Activated ras affinity precipitation assay

Activated ras affinity precipitation assay was performed as described previously, with slight modifications (28, 29). A GST fusion protein containing the RBD of raf-1 (aa 1–149 of raf-1), which binds only GTP-bound (activated) ras, was immobilized on glutathione-agarose beads (Upstate Biotechnology). After stimulating basophils (~5 × 10⁶ cells per condition), reactions were stopped by adding ice-cold PAG and microfuged for 5–10 s. The cell pellets were immediately lysed in ras affinity precipitation buffer (25 mM HEPES (pH 7.5), 2 mM EGTA, 150 mM NaCl, 10 mM benzamidine, 25 mM N-acetyl-D,L-Leucine, 50 mM Na₃VO₄, 1 mM NaF, 5 mM leupeptin, 1 mM Na₃VO₄, 1% Nonidet P-40, and 1% PMSF). Clarified lysates were incubated with the GST-RBD beads (5 µl/sample) for 1 h at 4°C with rocking. The GST-RBD beads were washed three times with ras affinity precipitation buffer. Bound proteins were eluted by boiling in ESB for 5 min. Affinity-precipitated ras was detected by immunoblotting with anti-ras mAb.

DNA affinity adsorption for STAT5

DNA affinity purification was described as previously, with modifications (30). FC-rk1-GAS probe (5′-GTATTCCACCAGAAAAGG AAC) with 3′-terminal biotinylination and its complementary strand were synthesized (BioSource International). After annealing of the two single-strand oligonucleotides, the double-stranded oligonucleotide was incubated with streptavidin-conjugated agarose beads (Pierce, Rockford, IL) for 1 h at 4°C and washed twice with CLB buffer. After stimulating basophils, the reaction was stopped by adding ice-cold PAG and cell suspensions were microfuged. The cell pellets were immediately lysed in CLB. Lysates were precleared with agarose beads for 1 h at 4°C to remove any nonspecific binding to the beads. The lysates were then incubated with GAS probe beads for 1 h at 4°C. The beads were washed three times with CLB buffer and the affinity-adsorbed protein was eluted by boiling in ESB for 5 min. Electrophoresis, transfer, and immunoblotting was performed as described above.

Intracellular Ca²⁺ concentrations ([Ca²⁺]i) measurements

Basophils were labeled with 1 µM fura-2 AM for 20 min at 37°C in RPMI 1640 containing 2% FCS (300,000–500,000 cells in 200 µl; purity >90%). After washing once with 200 µl of PAG, the cells were resuspended in PAGCM buffer containing 150 mM NaCl, 5 mM MgCl₂, 10 mM glucose, 20 mM HEPES (pH 7.5), and 1 µM ionomycin and stimulated with IL-3 for a single ratio measurement of intracellular Ca²⁺ concentration. After a 10 s challenge, cells were stimulated with 2 mM CaCl₂, and [Ca²⁺]i levels were acquired. The cells were challenged with 1 µM of ionomycin in buffer. Data were then acquired for 50–150 frames at intervals of 1–10 s to determine the subsequent [Ca²⁺], response.
Characteristics of Priming by IL-5 and NGF

**IL-5 or NGF induce acute priming but not late priming.** As noted above, IL-3 primes basophils so that they secrete LTC4 when stimulated with C5a. Treatment for 15 min or 24 h results in changes in basophil behavior but the mechanisms for each phase are different (23). Thus, we examined whether IL-5 or NGF would also induce biphasic priming of basophils. IL-3-induced changes were used as a positive control. As shown in Fig. 1A, treatment with IL-5 or NGF for 15 min significantly enhanced C5a-induced LTC4 release in the concentration range of 1–100 ng/ml. The magnitude of LTC4 release in cells stimulated with C5a was similar whether the cells were incubated with IL-3 or IL-5, although NGF was slightly less effective, as reported previously (3, 4). In contrast with the acute effect, significant enhancement of C5a-induced LTC4 release was not observed after 18-h pretreatment with IL-5 or NGF, whereas marked LTC4 release was caused by C5a from the cells treated with IL-3. The magnitude of the acute effect for IL-5 (10–100 ng/ml) and IL-3 (10 ng/ml) was similar so we examined the relative potency of the two cytokines to acutely prime the cell for LTC4 release. As shown in Fig. 2, the EC50 for the priming effect by IL-3 was 0.2 ng/ml, as reported previously (23), whereas that for IL-5 was higher (EC50 = 1 ng/ml).

**Expression of FceRIβ.** We have recently reported that IL-3 induces marked changes in both mRNA and protein for the β subunit of the high-affinity IgE receptor FcεRI (34). We examined FceRIβ mRNA in basophils stimulated with 10 ng/ml IL-3, IL-5, or NGF. Fig. 3 shows that both IL-3 and IL-5 induced similar levels of mRNA while NGF had no effect. In one of these experiments, we examined 30 and 100 ng/ml NGF, but these concentrations were also without effect (data not shown).

**Basophil survival.** It has been known for sometime that IL-3 prolongs in vitro survival of basophils (35, 36). We examined the survival value of IL-5 and NGF; the results of these experiments are shown in Table I. In pilot studies, earlier times were examined, and the data were consistent with the data in Table I, but the 3-day time point provided better discrimination. Notably, NGF was the poorest at promoting survival, which in these studies was most effectively measured by the viability of the cells rather than their recovery. Cell recovery paralleled the viability but the difference between medium alone and IL-3 was modest (60% of the cells placed in culture where recovered in IL-3 cultures and 52% were recovered from cultures of medium alone). An assessment of viability with either erythrocin B or propidium iodide yielded similar results. Notably, the difference in viability between NGF and medium alone was significant for either method, p < 0.0001 and p = 0.033 for erythrocin B and propidium iodide, respectively. (IL-3 and IL-5 were also statistically different from medium alone.)

**Signaling following IL-3**

**ERK pathway.** Before doing comparative studies of the three cytokines, we first examined in greater detail some of the signaling

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**LTC4 measurements**

Fifty thousand basophils were stimulated in a final volume of 100 μl of RPMI 1640 containing 2% FCS and 25 μg/ml gentamicin at 37°C in a 5% CO2 incubator. The reactions were terminated with 900 μl of ice-cold PAG-EDTA and the cells were then centrifuged in a microfuge at 14,000 rpm for 10 s. A RIA was performed using 100 μl of supernatant to determine LTC4 levels as previously described (1, 32). Each condition tested was performed in duplicate.

**Flow cytometry**

Basophil viability was assessed by staining with propidium iodide (2 μg/ml) for 1 min before analysis by flow cytometry. The percent positive cells (nonviable) was determined by setting a gate whose minimum value was at the point where >98% of unstained cells were excluded. The maximum of the gate was set to include the remaining range of fluorescence. Cells falling within this analysis region were deemed nonviable.

**Real-time PCR**

Purified basophils were cultured for 4 h with or without cytokines and subjected to total RNA extraction using the RNAzol method according to the manufacturer’s instructions. Samples were subjected to reverse transcription and real-time PCR amplification with primers specific to the human FceRIβ gene (33) (5'-CCA-GGA-AGT-ATC-TTG-AGG-CAG-CTC-3'; 5'-TCA-AAA-CTG-TCA-GGCC-ATG-TAT-GC-3') along with an internal reporter construct containing 6-carboxyfluorescein and 6-carboxytetramethylrhodamine fluorochromes (5'-FAM-TTG-AGG-TGC-GCC-TCA-TCC-CCA-CC-TAMRA-3'; BioSource International) using the TaqMan kit (PerkinElmer, Norwalk, CT). Reaction conditions were as follows: reverse transcription at 48°C for 10 min; 40 cycles at 95°C for 15 s/60°C for 1 min. Real-time PCR monitors the time course of the reaction by the appearance of unquenched fluorescence in the sample tubes. The number of cycles required to obtain fluorescence values 10 times the SD of the first 15 cycles of fluorescence was calculated for each sample. Previous studies established that for this probe and primer combination (using these reaction conditions), 2-fold dilutions result in 1.02 ± 0.15 cycle differences. Therefore, the relative expression of FceRIβ mRNA was calculated as 2^ΔΔCt (cycle difference between two samples).

**Results**

**Functional characteristics**

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steps that could be surmised from other cell models to follow stimulation with IL-3. In pilot studies, we first examined tyrosine-phosphorylated proteins following activation with IL-3, processed by generating whole-cell lysates and Western blotting with anti-phosphotyrosine Ab. A transient increase in tyrosine phosphorylation of several proteins (≈130, 120, 95, 70, 55, and 42 kDa) was observed (data not shown). Reblotting with specific Abs suggested these proteins were ERK1 (p44 MAPK) and ERK2 (p42 MAPK), as described previously (23, 37), and IL-3R (βc), JAK2, STAT5, SHP2, and Shc at ≈130, 120, 95, 70, and 55 kDa, respectively.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>IL-3</th>
<th>IL-5</th>
<th>NGF</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocin B</td>
<td>94 ± 1</td>
<td>82 ± 5</td>
<td>76 ± 7</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>96 ± 1</td>
<td>82 ± 4</td>
<td>79 ± 4</td>
<td>67 ± 5</td>
</tr>
</tbody>
</table>

*Shown is the viability of purified basophils cultured with IL-3 (10 ng/ml), IL-5 (10 ng/ml), NGF (10 ng/ml), or medium alone for 3 days. Two methods of assessment were used, erythrocin B (n = 9) or propidium iodide (by flow cytometry) (n = 7). The basophils were cultured in polypropylene microtubes and only resuspended prior to sampling for either the erythrocin B or propidium iodide measurement (i.e., there was no centrifugation step).

With these results as a basis for further study, we examined these signaling elements in greater detail using immunoprecipitation. From extensive studies in other cell models, JAK2 phosphorylation of IL-3R βc and other cytoplasmic signaling proteins are likely to be important in transmitting signals for cellular functions (38). Not surprisingly, as shown in Fig. 4, A and B, both JAK2 and IL-3 Rβc were indeed tyrosine phosphorylated upon activation with IL-3. A maximum phosphorylation of JAK2 and IL-3 Rβc was observed at 5 min and had decreased to near basal levels by 45 min.

In studies of other cells have also suggested that the activation through βc (by IL-3, IL-5, and GM-CSF) that leads to JAK2 activation results in the phosphorylation of Shc (38). Phosphorylated Shc can then bind to Grb2/Sos dimers to recruit them to the cell surface. The GDP/GTP exchange factor, Sos, in the assembled complex interacts with p21ras and initiates the swap of GDP for GTP in ras. Since we have previously demonstrated that acute enhancement of C5a-induced LTC4 release from basophils requires ERK and cPLA2 phosphorylation (25, 27), an examination of these elements was warranted. Thus, phosphorylation of Shc and association of Grb2 and Shc induced by IL-3 were examined. As shown in Fig. 4C, Shc was also transiently phosphorylated by exposure of the cells to IL-3. Similar to phosphorylation of βc and JAK2, a maximum phosphorylation of Shc was observed at 5 min and had decreased to near basal levels by 45 min. To determine whether Grb2 and Shc were associated following stimulation, Grb2 was immunoprecipitated from cell lysates and subsequent Western blotting was performed with anti-Shc. Tyrosine-phosphorylated 52- and 70-kDa proteins were coimmunoprecipitated by anti-Grb2 Ab (Fig. 4D) and their presence was transitory, i.e., present at 5 min and not present after 45 min. The tyrosine-phosphorylated 52- and 70-kDa bands were found to colocalize with bands following blotting with anti-Shc and anti-SHP2 Ab, respectively. These results suggest that both phosphorylated proteins were associated with Grb2. To verify the tyrosine phosphorylation of SHP2, immunoprecipitation with anti-SHP2 Ab and immunoblotting with anti-phosphotyrosine Ab was performed. As shown in Fig. 4E, SHP2 was also transiently phosphorylated following stimulation with IL-3. It should be noted that SHP1 was not detected in the proteins immunoprecipitated with anti-Grb2 and tyrosine phosphorylation of SHP1 following activation with IL-3 was not detected (although SHP1 is expressed in basophils (39), and data not shown).

Next, we examined activation of p21ras using affinity precipitation by a GST fusion protein containing the RBD of raf, since
activated ras protein (ras-GTP) bound to RBD (see details in Materials and Methods). As shown in Fig. 4F, the mass of ras (GTP-bound form) was markedly increased following activation with IL-3. This result also demonstrates that activation of p21sarp following stimulation with IL-3 was transient. Although our previous studies examined phosphorylation of ERKs (23), we verified that ERK phosphorylation was also transient in the current studies (data not shown).

STATS/JAK2 pathway. Phosphorylation of STAT5 (by JAK2) is likely to be important in transmitting signals from the cell surface to the nucleus (38). Therefore, IL-3-induced phosphorylation of STAT5 was examined. As shown in Figs. 5A and 6, STAT5 was phosphorylated following activation with IL-3, peaking from 5 to 15 min. This phosphorylation was decreased to 46% of maximum by 45 min, suggesting that activation of STAT5 is more sustained than that of JAK2 but nevertheless transient relative to some of the 24-h or multiday priming effects we observe with IL-3. These kinetics apply whether the cells are stimulated in PAGCM or RPMI 1640 (+2% FCS; data not shown). It is worth noting that this difference in behavior between JAK2 and STAT5 kinetics (Fig. 6) also translates to the concentration dependence of the response. Fig. 5C shows that stimulation with 1 ng/ml IL-3 results in a response that is <10% of the response at 10 ng/ml for both phosphorylation of IL-3β receptor and JAK2 while the phosphorylation of STAT5 at 1 ng/ml is ≈80% of the response observed using a concentration of 10 ng/ml. This concentration dependence varies significantly among basophil preparations but the relative relationship between JAK2 and STAT5 phosphorylation persists. Activated (phosphorylated) STAT5 dimerizes via association of one partner’s SH2 domain to the other partner’s phosphorylated tyrosine residue. The dimer, in turn, gains the ability to bind to DNA. The ability of STAT5 to bind to a relevant DNA oligonucleotide probe (a nucleotide sequence bound by most of the STAT family of proteins) was examined using the GAS probe (a nucleotide sequence induced by IL-3 in human basophils. Basophils were stimulated with various concentrations of IL-3, the reactions stopped at 5 min and processed as above, for Western blotting with anti-phosphotyrosine Ab. Three lysates were generated for each condition and immunoprecipitated with either anti-IL-3β, JAK2, or STAT5. The data shown are representative of three experiments.

FIGURE 4. Tyrosine phosphorylation of early signaling elements, or ras activation, induced by IL-3 in human basophils. Basophils were incubated with or without IL-3 (10 ng/ml) for the times indicated. Reactions were stopped with the addition of ice-cold PAG, the cells were centrifuged, and clarified lysates were immunoprecipitated with anti-IL-3Rβ Ab (A), anti-JAK2 Ab (B), anti-shc (C), anti-Grb2 (D), or anti-SHP2 (E) Ab. RBD-GST agarose affinity adsorption was used for the lysates shown in F. The immunoprecipitated proteins were subjected to Western blotting analysis with the indicated Abs as described in Materials and Methods. The membranes shown in F were stripped and reblotted with anti-GST Ab. The anti-GST Ab indicates that the RBD-GST fusion proteins (predicted molecular mass: ~45 kDa) were equally loaded. Each Western blot shown is representative of three separate experiments.

FIGURE 5. Phosphorylation of STAT5 (A) and its binding to DNA probe (B) induced by IL-3 in human basophils. Basophils were stimulated with or without IL-3 (10 ng/ml) for the times indicated (A) or for 10 min (B). A, Reactions were stopped with the addition of ice-cold PAG, the cells were centrifuged, and clarified lysates were immunoprecipitated with anti-STAT5 Ab. The immunoprecipitated proteins were subjected to immunoblotting with anti-phosphotyrosine Ab as described in Materials and Methods. The same membranes were stripped and reblotted with anti-STAT5 Ab. The immunoblotting shown is representative of three separate experiments. B, STAT5 in the clarified lysates was adsorbed on either a GAS probe/bead or beads containing bound anti-STAT5 Ab. The precipitated proteins were subjected to immunoblotting with anti-phosphotyrosine Ab as described in Materials and Methods. The same membranes were stripped and reblotted with anti-STAT5 Ab. The data shown are representative of two separate experiments. C, Basophils were stimulated with various concentrations of IL-3, the reactions stopped at 5 min and processed as above, for Western blotting with anti-phosphotyrosine. Three lysates were generated for each condition and immunoprecipitated with either anti-IL-3β, JAK2, or STAT5. The data shown are representative of three experiments.
precipitation following activation with IL-3, suggesting that phosphorylated STAT5 is capable of binding to DNA. Fig. 6 summarizes the kinetic results for some of these signaling elements.

**Drug effects on IL-3-mediated signaling events.** To partially test the cause and effect relationships between some of these signaling elements, we examined the effects of staurosporine (a protein kinase inhibitor) and Ro-31-8220 (a protein kinase C (PKC)-specific inhibitor) on IL-3-mediated phosphorylation of JAK2, Shc, STAT5, and ERKs. We have previously demonstrated that 1 μM Ro-31-8220 completely inhibits PMA-mediated events, and specifically the PMA-induced activation of the p21ras pathway, in human basophils (21, 27). As shown in Fig. 7A, phosphorylation of JAK2, Shc, STAT5, and ERKs induced by IL-3 was inhibited by staurosporine but not Ro-31-8220. In data not shown, 1 μM staurosporine also inhibited IL-3-induced expression of FcεRIβ mRNA. Not shown in the figure are experiments which indicate that the putative JAK2 kinase inhibitor AG490 does not alter either JAK2 or STAT5 phosphorylation. Fig. 7B demonstrates that stimulation with IL-3 does not cause phosphorylation of syk and that SHP2 phosphorylation occurs with IL-3 stimulation but not anti-IgE Ab. We have previously demonstrated that activation of the ERK pathway following stimulation with anti-IgE Ab is sensitive to the phosphatidylinositol 3-kinase (PI3) inhibitor LY294002. The results of IL-3-induced ERK phosphorylation in the presence of LY294002 have been variable. Fig. 7C shows that there was little or no inhibition of ERK phosphorylation by 10 μM LY294002 (a concentration which completely inhibited IgE-mediated ERK phosphorylation). But, we have found ~50% inhibition of IL-3-mediated ERK phosphorylation in the presence of 30 μM LY294002 (data not shown). Wortmannin also had only modest effects. However, we have not noted any inhibition of IL-3-induced STAT5 phosphorylation by LY294002, even at 30 μM (Fig. 7C). The putative lyn kinase inhibitor, PP1, effectively inhibited ERK phosphorylation induced by anti-IgE Ab (as shown previously) but not IL-3 (Fig. 7D).

**Comparative signaling**

**ERK pathway.** The above studies indicate that IL-3 signals through at least two pathways that regulate transcription factors, one which leads to ERK phosphorylation (and cPLA₂ phosphorylation)—the shc/Grb2/SOS/ras/ERK pathway—and one which

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**FIGURE 6.** Kinetics of phosphorylation of JAK2, IL-3Rβ, shc, and STAT5 induced by IL-3 in human basophils. Quantitative analyses of digitized images of the three experiments of phosphorylation of JAK2, IL-3Rβ, shc, ERK, and STAT5 induced by IL-3 (from Figs. 4 and 5 and data not shown).

![Graph](image-url)
equal protein loading for each lane because the sum of phosphorylated and nonphosphorylated forms was equal in all lanes. We have found that on average, at the 5-min time point and using 10 ng/ml of each of the cytokines, that IL-5 and NGF induce 0.56 ± 0.15 (n = 6) and 0.45 ± 0.20 (n = 9) of the phosphorylation observed following IL-3, respectively. The range of the latter ratio (NGF:IL-3) is marked, with a low of 0.05 and a high of 2.0. Not surprisingly, both IL-3 and IL-5 caused phosphorylation of Shc (data not shown). NGF also induced phosphorylation of Shc (Fig. 8B). These results suggest that activation of ERKs and cPLA2 may be responsible for the acute priming effect for LTC4 generation induced by IL-5 or NGF. Although the magnitude of ERK1/2 phosphorylation was similar for IL-3 and IL-5, the kinetic profile was somewhat different. As shown in Fig. 8C, IL-5- and NGF-induced ERK1/2 phosphorylation is more transient than that induced by IL-3. By 30 min, IL-5-induced ERK1/2 phosphorylation had returned to the basal level whereas IL-3-induced phosphorylation was still observed. Consistent with the kinetics of phosphorylation of ERK1/2, the priming effect of IL-5-, as assessed by enhanced C5a-induced LTC4 release, was also more transient than that caused by IL-3 (Fig. 8D). Data in Table II show that in basophils primed with all three cytokines, PD98059 (a MAPK kinase inhibitor) inhibited C5a-induced LTC4 release.

Comparative activation of JAK2 and STAT5. As expected for two cytokines that share a common β subunit, both IL-3 and IL-5 also activate JAK2 and STAT5 (Fig. 9, A and B). However, NGF does not appear to activate either JAK2 or STAT5 (Fig. 9, C and D). It is apparent that although NGF is capable of activating the ERK pathway, it often does so less effectively than IL-3. We considered the possibility that IL-3 appeared to induce phosphorylation of STAT5 because it generates a much stronger signal than NGF. To compare the relative strength of the signals, we titrated the IL-3 response with serial dilutions of IL-3 so that the ERK response matched the lesser ERK response induced by NGF. At this point of equivalence (generally occurring at 0.3 ng/ml IL-3), IL-3 continued to induce significant phosphorylation of STAT5 (data not shown).

Enhancement of the C5a-induced [Ca\(^{2+}\)]\(i\) elevation. For cells incubated with IL-3 for 18 h, the increased and sustained [Ca\(^{2+}\)]\(i\) elevation is possibly the new element which allows C5a to induce LTC4 release (15, 23). We examined the C5a-induced [Ca\(^{2+}\)]\(i\) elevation in basophils primed with all three cytokines, PD98059 (a MAPK kinase inhibitor) inhibited C5a-induced [Ca\(^{2+}\)]\(i\).  

### Table II. Effects of PD98059 on C5a-induced LTC4 release in basophils primed with cytokines

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>PD98059</th>
<th>% Inhibition (by PD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.6 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5a</td>
<td>2.0 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3 + C5a</td>
<td>39 ± 17</td>
<td>15 ± 7.3</td>
<td>70 ± 11(^{a})</td>
</tr>
<tr>
<td>IL-5 + C5a</td>
<td>25 ± 11</td>
<td>7.3 ± 5.8</td>
<td>81 ± 12(^{a})</td>
</tr>
<tr>
<td>NGF + C5a</td>
<td>24 ± 12</td>
<td>5.9 ± 3.0</td>
<td>83 ± 8.4(^{a})</td>
</tr>
</tbody>
</table>

\(^{a}\) Human basophils were preincubated with DMSO (0.1%) or PD98059 (100 µM) for 1 h, followed by an incubation with IL-3, IL-5, or NGF at a concentration of 10 ng/ml for 15 min, and then stimulated with C5a (50 ng/ml) for 30 min. Reactions were terminated by the addition of ice-cold PAG-EDTA, and the cells were centrifuged. Supernatants were collected and analyzed for LTC4 (pmol/10\(^{6}\) basophils). Values are mean ± SEM of three experiments.

\(^{b}\) Statistically significant inhibition from LTC4 release in the cells treated with DMSO control (p < 0.05, as determined by paired t test).
FIGURE 9. Phosphorylation of early signaling elements by IL-3, IL-5, or NGF. A, Basophils were incubated with or without IL-3 (10 ng/ml) or IL-5 (10 ng/ml) for the times indicated. Reactions were stopped with the addition of ice-cold PAG, the cells were centrifuged, and clarified lysates were immunoprecipitated with anti-JAK2 Ab or anti-STAT5. B, A similar experiment shown for stimulation with IL-3 and 10 ng/ml NGF. The results are representative of three separate experiments.

Discussion

In the current study, we first examined the relative ability of three cytokines known to acutely prime basophils for C5a-induced LTC4 release for their ability to alter other, later aspects of basophil function. First, only IL-3 also enhanced C5a-induced LTC4 release when the preincubation with the cytokines was on the order of 18 h. Second, IL-3 and IL-5 both induced the increased expression of mRNA for FcεRIβ while NGF did not. Third, IL-3 was best at maintaining the viability of basophils in culture, with IL-5 and NGF allowing better viability than medium alone but significantly less than with IL-3. Of these observations, the most interesting dichotomy for two cytokines that were capable of priming basophils for C5a-induced LTC4 release was between IL-3 and NGF and the marked difference in their ability to induce mRNA accumulation for FcεRIβ. It was in this context that we were most interested in the signaling differences for the two cytokines. The expectations for signaling with IL-5 were that it would be a weaker version of the signaling for IL-3, since these two share a common β subunit and it is known that there may be up to 10-fold fewer IL-5 α subunits expressed on the basophils surface than IL-3α (40).

The difference in potency between IL-3 and IL-5 and the otherwise similar affinity of the two cytokines for their respective receptors suggests that any quantitative difference in signaling for these two cytokines is a reflection of the differences in receptor density. An important caveat to this statement is our lack of knowledge concerning the quality of the recombinant cytokine preparations. It is unclear whether mole for mole, the commercial preparations of these two cytokines could be considered equal in ability to bind to their respective α subunits. For example, we have found that there is significant variation in the potency of IL-3 preparations even from the same manufacturer. However, under the best of conditions for both IL-3 and IL-5, there continues to be a difference in potency.

To understand the origins of the differences between IL-3 and NGF, we first examined some of the signaling steps well described in studies of cell lines. Because studies which examine cause and effect for signaling elements in human basophils are quite limited, it is necessary to interpret the basophil studies in light of what is known from studies of cell lines. The expectation was that IL-3 binding to its receptor and the subsequent receptor dimerization would result in activation of JAK2 kinase, most likely due to JAK2 transphosphorylation. JAK2 phosphorylation of IL-3Rβc and other cytoplasmic signaling proteins (Shc and STAT5) is likely to be important in transmitting signals for cellular functions (38). In this context, we found expected changes in these signaling elements in human basophils. If the cell line models are valid for human basophils, our results suggested that phosphorylation of JAK2 and Shc occurred, followed by association of Shc and Grb2 and ras activation leading to ERK activation for the priming effect that acutely alters LTC4 release. These activation events were transient and kinetically associated with ERK activation. These transient characteristics are then reflected in the transient acute priming by IL-3 for LTC4 release, as documented in previous studies (23). It seems likely that activation of TrkA receptors on basophils, by NGF also results in the phosphorylation of Shc by the intrinsic kinase activity of the TrkA receptor (6), which results in activation of the ERK pathway that is similar to that which is known with IL-3. NGF-mediated activation of the ras-ERK pathway has been reported in other cells (41–43). The strength of the initial signals is, on average, weaker for NGF and, like IL-5, the weaker activation results in a more transient response, both at the level of ERK phosphorylation and priming for C5a-induced LTC4 release.

An interesting aspect of the JAK2/STAT5 response is that a low JAK2 signal translates to a stronger STAT5 signal. This can be observed in the dose-response curve for IL-3, the comparison of IL-3 and IL-5, or the longevity of the STAT5 response relative to the JAK2 response. The data have the appearance of a signaling element that integrates the strength of the antecedent signal. This may come about because the opposing dephosphorylation reaction for STAT5 is weak or the STAT5 translocates out of its normal

FIGURE 10. The [Ca2+] response induced by C5a after pretreatment with IL-3, IL-5, or NGF (18 h). Basophils were incubated without (○) or with IL-3 (10 ng/ml, □), IL-5 (10 ng/ml, ○), or NGF (10 ng/ml, △) for 18 h, washed, and labeled with fura-2 AM. After washing, the cells were stimulated with C5a (50 ng/ml). The [Ca2+] response was analyzed as described in Materials and Methods. The results shown are representative of two separate experiments.
environment, something that would not be surprising for a transcription factor. It has been reported that JAK2 activation and its downstream events are susceptible to staurosporine (a general protein kinase inhibitor) (44, 45). In human basophils, staurosporine also inhibited phosphorylation of JAK2 and its putative downstream signaling events (phosphorylation of Shc, STAT5, and ERKs). In contrast, Ro-31-8220 (PKC-specific inhibitor) did not affect these events, indicating that a PKC is not responsible for these events. AG490 (tyrophostin B42) has been reported as a JAK2 inhibitor but we could not find evidence that it acted on JAK2 using concentrations as high as 100 μM. With respect to ERK phosphorylation, it is less clear how involved is a PI3 kinase since the selective inhibitor, LY294002, had variable and incomplete effects, and the sporadic inhibition observed only occurred at relatively high concentrations. Significant inhibition of IgE-mediated secretion and ERK phosphorylation occurs at 3–4 μM (IC50). On the other hand, we have also previously found that somewhat higher concentrations of LY294002 (an IC50 of ≈10 μM) inhibit the IgE-mediated elevation in cytosolic-free calcium. Nevertheless, the high concentrations required to observed any inhibition suggest possible nonselective effects of the drug. In contrast, LY294002 did not inhibit STAT5 phosphorylation, suggesting that this pathway is unaffected by PI3 kinase activity, a result that is perhaps not surprising given the relatively direct connection between JAK2 activation and STAT5 phosphorylation. Finally, although there are indications that lyn kinase plays a role in IL-5 priming of eosinophils (46), we could not detect an effect of PP1 on either the ERK or STAT5 pathways. In contrast, we have shown that it effectively inhibits a variety of IgE-mediated functions and signaling elements (47), data which are consistent with its ability to inhibit lyn kinase.

Phosphorylation of Shc is a critical step for ras activation since phosphorylated Shc can then bind to the Grb2-Sos complex to activate ras (48). It has been reported that lyn kinase can bind to the Grb2-Sos complex (49). We have previously demonstrated that human basophils express Sos2 but not Sos1 and that Sos2 protein is constitutively associated with Grb2.4 Sos2 protein is not phosphorylated or dissociated from Grb2 following activation with IL-3 in human basophils (data not shown). It has been reported that the role of SHP2 in cytokine receptor activation is context sensitive, in some cases acting as a positive regulator of cytokine signaling (50) and sometimes acting as a negative regulator (51). We have shown that SHP2 appeared to be phosphorylated on tyrosine in response to IL-3 and associated with Grb2 but have not determined whether this has a positive or negative influence on the course of the signaling events.

In contrast with the relative similarity of acute priming events, there were marked differences in the effects of these three cytokines on responses that could be considered to require a longer cascade of events. Table III summarizes these differences as well as the differences in signaling. Unlike the ability of IL-3 to augment the [Ca2+]i response that follows stimulation with C5a, and in particular to enhance the second phase of the [Ca2+]i response (15, 23), treatment with IL-5 and NGF was similar to culture without any cytokines. These results also support the hypothesis that the sustained (or enhanced) [Ca2+]i response facilitated by IL-3 is essential for its late priming effect (23). The absence of a late priming effect using NGF might not be surprising since the TrkA receptor may initiate signaling that is different from the signaling started by the β subunit of IL-3/IL-5. Indeed, these studies show that there is at least one signaling difference between the IL-3 receptor and TrkA receptors, the absence of JAK2 and STAT5 activation by NGF. However, IL-3 and IL-5 presumably operate through the same β subunit. This difference in JAK2 activation and the more transient nature of IL-5 acute priming suggests that the difference at 18 h may be the result of quantitative issues rather than qualitative aspects of signaling.

It is possible that the qualitative difference in the ability of IL-3 and IL-5 to induce increased mRNA for FcεRIβ compared with NGF is also the result of the inability of NGF to induce STAT5 activation. Unfortunately, we have yet to find a specific inhibitor that would allow us to demonstrate that FcεRIβ mRNA accumulation requires STAT5 activation. We did find that staurosporine inhibited both IL-3-induced STAT5 phosphorylation and FcεRIβ mRNA up-regulation, but this drug is so broad a kinase inhibitor that this result is difficult to interpret. An intriguing observation that places NGF in a somewhat different category is that it does alter basophil viability in a relatively long-term culture, albeit much less so than IL-3. In some experiments, IL-3 and NGF resulted in a similar retention of viability, indicating the close similarity in the effect of these two cytokines. To reflect this similarity, we assigned a single plus for both IL-5 and NGF in this category in Table III and a double plus for IL-3. Considering that there was a reasonable association between the activation of STAT5 and the ability of a cytokine to up-regulate FcεRIβ mRNA, the partial dissociation between STAT5 activation and survival (IL-3 vs NGF) is notable. When the data are viewed from the crude perspective of the plus-minus assignments in Table III, it appears that acute LTC4 priming and survival match ERK activation while FcεRIβ mRNA up-regulation matches STAT5 activation. However, the overnight enhancement of the cytosolic calcium response matches neither signaling pathway. It remains possible that the enhancement of the calcium response depends on ERK pathway activation but is more sensitive than survival to the relative strength of this activation. Alternatively, survival depends not on ERK activation but pathways not yet studied.

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