

Differences in proliferation of the hematopoietic cell line TF-1 and cytokine production by peripheral blood leukocytes induced by 2 naturally occurring forms of human IL-3

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Background: A naturally occurring polymorphism in the coding region of the human *IL3* gene leads to a change in amino acid residue 8 from proline to serine.

Objective: We sought to determine whether the 2 different forms of IL-3 varied in function. These different forms are available as recombinant proteins (recombinant human IL-3/proline 8 [rhIL-3/P8] and recombinant human IL-3/serine 8 [rhIL-3/S8]).

Methods: The erythroleukemic cell line TF-1 was incubated with varying concentrations of rhIL-3/P8 or rhIL-3/S8 to determine the capacity of each type of IL-3 to induce proliferation. Human leukocytes were primed with rhIL-3/P8 or rhIL-3/S8 for up to 24 hours and then stimulated with anti-IgE and assessed for leukotrienes (LTs), IL-4, and TNF- α .

Results: Proliferation of TF-1 cells was induced by both forms of IL-3 at 48 and 72 hours but to a greater degree by rhIL-3/P8. In contrast, the mean fold increase over control values of LT and IL-4 production was higher after priming the cells with rhIL-3/S8 versus rhIL-3/P8. Additionally, TNF- α production was greater (and reached significance only) for rhIL-3/S8. This activity was independent of IgE and thus directly stimulated by IL-3. Studies with basophil-enriched and basophil-depleted cell preparations revealed that LT production was evident only from the former and TNF- α only from the latter. **Conclusion:** We conclude that the 2 naturally occurring forms of human IL-3 have similar spectra of activities on cells with IL-3 receptors, but the 2 forms have reversed relative efficacies for promoting proliferation (rhIL-3/P8 > rhIL-3/S8) compared with priming or inducing mediator secretion (rhIL-3/S8 > rhIL-3/P8). (*J Allergy Clin Immunol* 2001;107:505-10.)

Key words: IL-3, polymorphism, priming, leukotrienes, TNF- α , IgE, proliferation, IL-3 receptor

IL-3 is a hematopoietic growth factor that stimulates the survival, proliferation, and differentiation of hematopoietic progenitor cells.^{1,2} IL-3 is also known to be a very potent primer of basophils in that exposure to IL-3 is asso-

Abbreviations used

LT: Leukotriene
rhIL-3/P8: Recombinant human IL-3/proline 8
rhIL-3/S8: Recombinant human IL-3/serine 8
SNP: Single nucleotide polymorphism

ciated with large increases in antigen- or anti-IgE-driven release of leukotrienes (LTs) and IL-4 from basophils.^{3,4}

The human *IL3* gene is located on chromosome 5q23-5q31 and encodes a protein of 152 amino acids, of which 19 are the signal sequence.^{5,6} Rosenwasser et al⁷ and Jeong et al⁸ have identified 2 single nucleotide polymorphisms (SNPs) located in the promoter region and the coding region, respectively, of the *IL3* gene. The SNP in the coding region (C-T at position +79) results in an amino acid change from proline to serine at residue 27 when including the leader sequence or residue 8 in the secreted form of human IL-3.

Variations in the amino acid sequence are potentially capable of altering the biologic activity of the expressed protein. Any such differences in activity associated with the 2 forms of IL-3 could provide a means of enhancing our understanding of the molecular interactions of IL-3 with its cell-surface receptors, could help explain gene by environment interactions in disease processes, and could have an effect on the therapeutic efficacy, potency, or both of side effect toxicity. To determine whether the single amino acid change affects the known biologic functions of IL-3, the 2 forms were examined here for differences in efficacy in relation to 4 different activities.

METHODS

Reagents

The recombinant human IL-3/proline 8 (rhIL-3/P8) was obtained from Biosource (Camarillo, Calif), and the recombinant human IL-3/serine 8 (rhIL-3/S8) was obtained from GIBCO BRL (Rockville, Md) and R&D (Minneapolis, Minn). Sequencing analysis performed by using the Applied Biosystem 477A Protein/Peptide sequencer at the University of Arizona, Biotechnology Facility, on the rhIL-3 from Biosource and GIBCO BRL verified the amino acid sequence differences provided on request by the manufacturers. They also verified the stock IL-3 concentrations as received (using an ABI 420A/H amino acid analyzer). Complete RPMI was pre-

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pared with RPMI-1640 supplemented with 5% heat-inactivated FCS, 1 mmol/L HEPES, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mmol/L L-glutamine (all from Life Technologies, Rockville, Md). Anti-IgE was obtained from Buehlmann Laboratories AG (Allschwil, Switzerland).

Leukocyte separation

Peripheral venous blood was obtained from healthy adult volunteers by a protocol approved by the university institutional review board. A leukocyte-enriched preparation was prepared by means of dextran separation. Briefly, 2.5 mL of 4.5% dextran (Buehlmann Laboratories AG) was added to 10 mL of blood. The cells were allowed to sediment for 90 minutes at room temperature. The leukocyte-rich plasma was centrifuged at approximately 130g for 15 minutes at 20°C to 30°C. After discarding the supernatant, the cell pellet was resuspended in as much complete RPMI as the initial volume of blood.

Basophil isolation

Basophil isolation was performed by means of negative selection with the MACS Basophil Isolation Kit from Miltenyi Biotec (Auburn, Calif), according to the manufacturer's instructions. Briefly, PBMCs were obtained by means of density gradient centrifugation over Lymphocyte Separation Medium (ICN Biomedicals Inc, Aurora, Ohio). For depletion of B cells, monocytes, natural killer cells, dendritic cells, early erythroid cells, platelets, neutrophils, eosinophils, and T cells from PBMCs, these cells were labeled with a cocktail of hapten-conjugated antibodies to CD3, CD7, CD14, CD15, CD16, CD36, and CD45RA, as well as anti-*HLA-DR* antibodies, and then mixed with MACS Micro Beads coupled to an anti-hapten mAb. The magnetically labeled cells were depleted by retaining them on a MACS column in the magnetic field, and the flow-through was collected as the cells of interest (basophil-enriched cells). The purity of the basophils in 2 such preparations was approximately 73% and 87%. Contaminating cells included lymphocytes, eosinophils, and neutrophils. The cells retained on the column were eluted outside the magnetic field and were examined for secretion in the same way as the basophils.

Priming of cells with IL-3

Serial dilutions of the 2 forms of IL-3 were made in complete RPMI. Cells were incubated for 24 hours at 37°C with each form of IL-3 (10^{-8} , 10^{-10} , and 10^{-12} mol/L final concentration) or control RPMI. Anti-IgE (Buehlmann Laboratories AG) was diluted in complete RPMI, and anti-IgE (undiluted or a 1:3 dilution) or complete RPMI was added to the cell suspensions described above and incubated for 40 minutes at 37°C water bath. After the incubation, the samples were centrifuged for 3 minutes at approximately 1000g at 4°C, and supernatants were harvested and then stored at -20°C until assayed.

Measurement of LTs, IL-4, and TNF- α

Production of LTC₄, LTD₄, and LTE₄ were measured by using the Buehlmann CAST-ELISA Kit (Buehlmann Laboratories AG). Specificity of the mAb is equivalent for these LTs. IL-4 and TNF- α were measured with HS ELISA Kits from R&D Systems, according to the manufacturer's instructions. Positive and negative control supernatants were run with each assay. Each of these assays has been exhaustively examined for cross-reactivity, as reported by the manufacturer.

Assay of cell proliferation

Cell proliferation was examined by using the Biotrak cell proliferation ELISA system, version 2, from Amersham Pharmacia Biotech (Piscataway, NJ), according to the manufacturer's instructions. TF-1 cells were obtained from American Type Culture Col-

lection (ATCC, Manassas, Va). They were grown initially in complete growth medium containing GM-CSF (3.75 ng/mL) from R&D Systems at 37°C in a 5% CO₂ incubator, according to the method of Kitamura and colleagues.⁹ Cells were washed 3 times in HBSS (Life Technologies), resuspended in GM-CSF free culture medium, and incubated at a final concentration of 10^4 cells per well in a 96-well microtiter plate. After incubating the cells (in triplicate) for 64, 40, and 16 hours in the presence or absence (10^{-8} , 10^{-10} , and 10^{-12} mol/L) of rhIL-3/S8 or rhIL-3/P8 5-bromo-2'-deoxyuridine was added to each well (final concentration, 1 mmol/L 5-bromo-2'-deoxyuridine), and the incubation continued for 8 hours.

Statistical analysis

To quantify the effect of stimulation with the 2 forms of IL-3 on LT, IL-4, TNF- α , and proliferation, we calculated the ratio of stimulation effect to control effect at each concentration of IL-3. For LT, IL-4, and proliferation, the ratios were normally distributed. For TNF- α , the natural logs of the ratios were normally distributed. Mean fold increase in LT, IL-4, TNF- α , and proliferation over control at each concentration of IL-3 for the 2 forms (rhIL-3/S8 and rhIL-3/P8) were compared by using a paired *t* test.

RESULTS

Potential alterations in the biologic activity for the 2 forms of IL-3 were examined in 4 ways. First, assessment of IL-3 activity involved comparing the capacities of rhIL-3/S8 and rhIL-3/P8 to promote proliferation of TF-1 cells, an erythroleukemic cell line that is completely dependent on the cytokines IL-3 or GM-CSF for growth. Cells were initially grown in GM-CSF, washed free of GM-CSF, and assessed for proliferation after addition of rhIL-3/S8 or rhIL-3/P8. Cells incubated with both forms of IL-3 showed enhanced proliferation at 48 and 72 hours. The induction for rhIL-3/P8 was greater at 72 hours than that for rhIL-3/S8 at 10^{-8} mol/L ($P = .063$) and 10^{-10} mol/L ($P = .017$, Fig 1). A 48-hour incubation with rhIL-3/P8 also resulted in significantly more proliferation than with rhIL-3/S8 at 10^{-10} mol/L (data not shown). No proliferation was evident at the 24-hour time point.

The second assessment addressed the capacity of rhIL-3/S8 and rhIL-3/P8 to prime leukocytes for enhanced release of LTs. Levels of LTs were measured in supernatants of dextran-separated leukocytes with anti-IgE stimulation. Fig 2 shows the mean fold increase over control (without IL-3) of LT levels in supernatants after priming dextran-separated cells from 8 subjects with 3 different concentrations of the 2 forms of IL-3. Cells primed with rhIL-3/S8 produced significantly higher amounts of LTs compared with control and compared with rhIL-3/P8 at both 10^{-8} and 10^{-10} mol/L. Cells primed with rhIL-3/P8 showed a significant increase in LTs only at 10^{-8} mol/L. The production of LTs was totally dependent on stimulation with anti-IgE and was minimal at 24 hours (data not shown).

The third assessment addressed the capacity of the 2 forms of IL-3 to prime the leukocyte preparations to produce IL-4 in response to anti-IgE. Fig 3, A, shows IL-4 production after priming cells from 3 different donors with IL-3 for 1 hour and then stimulating with anti-IgE. IL-4 production after priming with rhIL-3/S8 was mod-

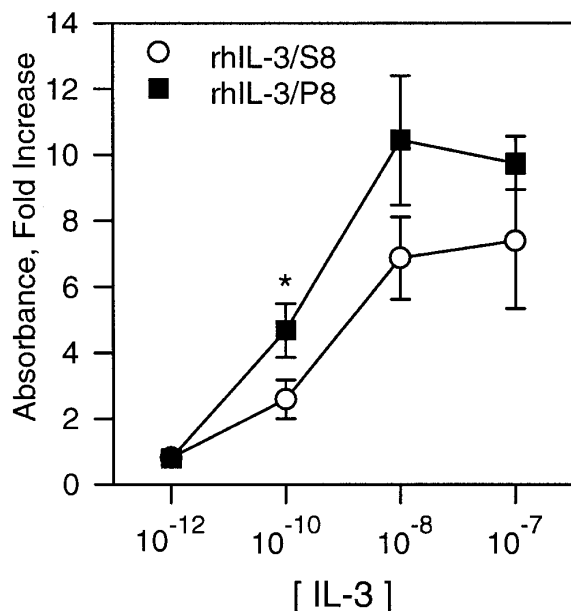


FIG 1. The rhIL-3/P8 form of IL-3 (squares) induces enhanced proliferation of TF-1 cells compared with rhIL-3/S8 (circles). TF-1 cells were cultured for 72 hours in triplicate. 5-Bromo-2'-deoxyuridine was added 8 hours before harvest. The number of experiments used at each concentration of IL-3 were 4 for 10⁻⁷ mol/L, 6 for 10⁻⁸ mol/L, and 7 for 10⁻⁷ mol/L and 10⁻¹² mol/L. Absorbance units for control samples were 0.037 ± 0.006. *Significant difference in the 2 forms. All values for each form are significantly greater than control values.

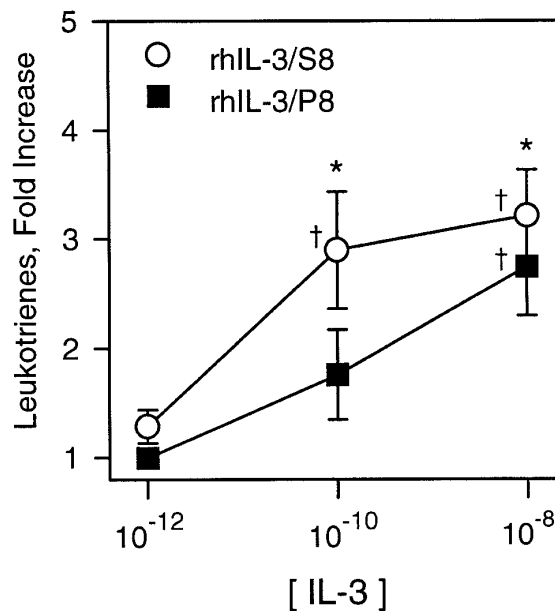


FIG 2. Human leukocytes primed with rhIL-3/S8 produce greater amounts of LTs compared with rhIL-3/P8. Dextran-separated cells were primed for 24 hours and stimulated with anti-IgE for 40 minutes before harvest. Each point represents the mean fold increase in IL-3-treated samples over control values (control RPMI) ± SEM for leukocytes from 8 donors. The production of LTs was undetectable without stimulation with anti-IgE (data not shown). *Significantly different amounts of LTs for the 2 forms of IL-3. †Significant difference from control values.

est and evident only at 10⁻⁸ mol/L. The rhIL-3/P8 did not prime significantly for IL-4 production. Priming for 4 hours (Fig 3, B) was significant only for rhIL-3/S8 and evident at both 10⁻⁸ and 10⁻¹⁰ mol/L. Priming for 24 hours (Fig 3, C) resulted in a very modest increase in IL-4 production for both forms of IL-3, which reached significance only for rhIL-3/P8. The IL-4 production was totally dependent on stimulation with anti-IgE (data without anti-IgE not shown).

The fourth assessment compared the capacities of rhIL-3/P8 and rhIL-3/S8 to stimulate leukocytes for the production of the cytokine TNF-α. Fig 4, A, shows TNF-α levels in supernatants from leukocyte-enriched cell populations generated by the same protocol as those assessed above for LTs. TNF-α was detectable in cultures treated with rhIL-3/S8 (significant at 10⁻⁸ mol/L and borderline at 10⁻¹⁰ mol/L) and was independent of anti-IgE (Fig 4, B). Cells stimulated with rhIL-3/P8 appeared to show enhanced TNF-α production, but the levels were not significantly greater than control over the concentration range used.

To investigate the source of TNF-α in the mixed-cell population, leukocyte preparations were separated into basophil-enriched and basophil-depleted populations and stimulated as in Fig 4. TNF-α was detectable in the basophil-depleted cell preparation stimulated with rhIL-3/S8 but not in the basophil-enriched preparation (n = 2, data not shown). As expected, given the dependence of LT production on anti-IgE, LTs were detectable only in the basophil-enriched population.

It is known that IL-3 enhances cytokine production by LPS-stimulated macrophages.^{10,11} To test whether the TNF-α production noted above might have involved LPS stimulation (by unintentional contamination), polymyxin B in different concentrations was added to the samples stimulated with 10⁻⁸ mol/L rhIL-3/S8 and rhIL-3/P8. Fig 5 shows a decrease in TNF-α production on the addition of polymyxin B, but the decrease also affected control samples, and the fold increase over control was similar to that in Fig 4, A and B.

DISCUSSION

IL-3 is known to be a hematopoietic growth factor and to have additional effects on mature cells. We provide data herein to show that 2 naturally occurring forms of human IL-3 differ at least quantitatively in initiating several biologic activities. The 2 forms of the protein are the result of a missense SNP in the coding region of the *IL3* gene (C vs T at position +79 from the open-reading frame, corresponding to proline vs serine, respectively) at position 27 when numbered as the immediate product of the gene (which includes the leader sequence) and is equivalent to position 8 in the mature product after cleavage of the leader sequence. Homozygotes for the T allele (serine) make up to 5% of the population in contrast to 60% homozygosity for the more common C allele (proline).⁸ Another SNP has been reported for the *IL3* gene at nt-68 in the promoter region. This SNP is in almost complete

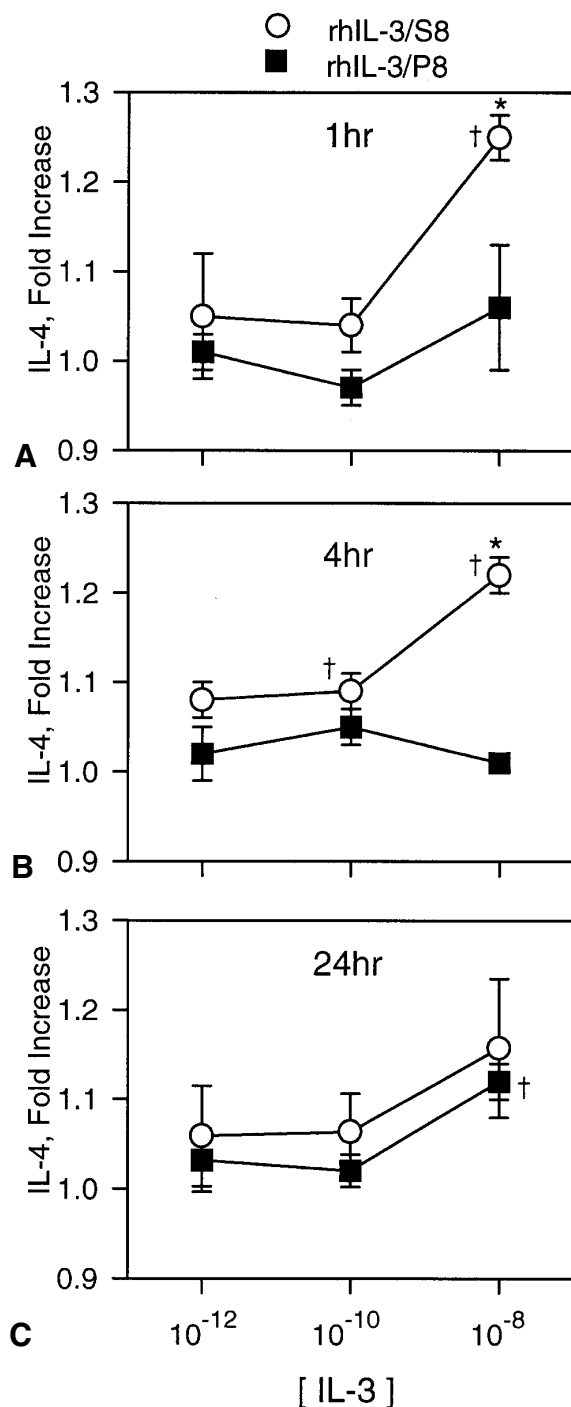


FIG 3. Human leukocytes primed with rhIL-3/S8 produce greater amounts of IL-4 compared with rhIL-3/P8. Dextran-separated cells were primed for 1 (A), 4 (B), or 24 (C) hours and stimulated with anti-IgE for 40 minutes before harvest. Each point represents the mean fold increase over control (control RPMI) $\pm$ SEM of IL-4 production for 3 different donors. *Significant difference between the 2 forms of IL-3. †Significant difference from control values.

linkage disequilibrium with that at +79.⁸ Further studies are needed to determine whether this second SNP affects gene expression and thus the amount of protein secreted.

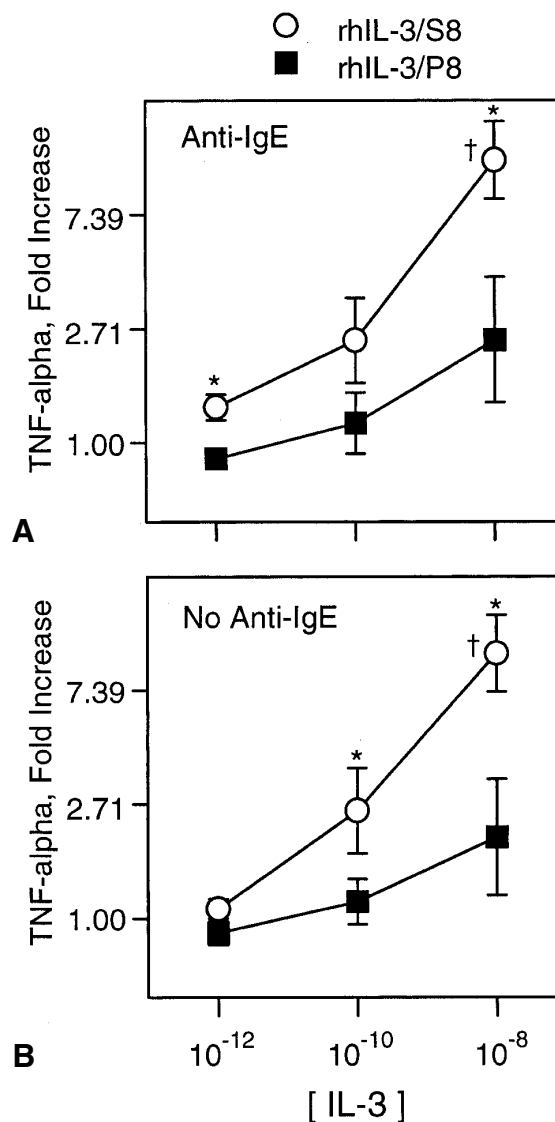


FIG 4. IL-3 directly induces TNF- α production from human leukocytes. Cells were stimulated with (A) or without (B) anti-IgE. Each point represents the mean fold increase over control (control RPMI) $\pm$ SEM of TNF- α production for 6 donors. Values for rhIL-3/P8 were not different from control values. The data show that the production of TNF- α was not dependent on stimulation with anti-IgE (B). *Significant differences between the 2 forms of IL-3. †Significant difference from control values.

The 2 naturally occurring forms of IL-3 differ in their capacity to induce proliferation of TF-1 cells with rhIL-3/P8, being more potent than rhIL-3/S8. Our data suggest that these 2 forms of IL-3 interact differently with the IL-3 receptor and may thus initiate some differences in the spectrum of intracellular pathways activated or different levels of activation of the same pathways. Indeed, studies with experimentally mutated and truncated forms of IL-3 have shown that the capacity to induce proliferation differs among the muteins.^{13,14} The naturally occurring genetic variants studied herein were not

reported. Given that both forms can induce proliferation and that an N-terminally truncated mutein of IL-3 missing the first 13 amino acids is also capable of inducing proliferation, this region of the first α -helix is not critical to the ligand-receptor interactions required to initiate the cascade of events leading to proliferation. Nevertheless, receptor ligand interactions of this region may influence that initiation. The amino acid in position 8 does appear to be in the first of the 4 α -helices purported to be present in native IL-3 on the basis of analogy and homology with other members of the species, such as GM-CSF.¹⁵ Computer-based docking studies with putative structures for IL-3 and its receptor also imply that amino acids in the first α -helix (helix A) appear to be in close proximity to the α chain of the IL-3 receptor.¹⁶ Thus the location of the naturally occurring allelic alteration is consistent with the possibility of an alteration in degree of activation but not with a complete loss of function. Whether the more rare rhIL-3/S8 gene variant is associated with alterations in phenotypic characteristics related to hematopoiesis remains to be determined.

The 2 forms of rhIL-3 show a reversal of relative potency for affecting mediator release from mature blood cells compared with the stimulation of proliferation of the erythroleukemic TF-1 cells. One of these activities is the capacity to prime basophils for antigen or anti-IgE stimulation leading to release of preformed (eg, histamine) and newly synthesized mediators (eg, LTs) and cytokines,^{17,18} reactions thought to be central participants in allergic asthma and allergy. We herein demonstrate that there was a greater release of LTs and IL-4 after priming blood leukocytes with rhIL-3/S8 compared with rhIL-3/P8. Basophil isolation experiments and the dependence on anti-IgE stimulation indicated that basophils appear to be responsible for the production of LTs.

TNF- α was also measured in the supernatants of leukocytes treated with the same protocol as that for LTs and IL-4. We demonstrated here that the leukocytes released TNF- α on stimulation with IL-3 independent of anti-IgE and, to a greater degree, with the rhIL-3/S8 versus the rhIL-3/P8 form. Although basophils have been shown capable of producing TNF- α ,^{19,20} the TNF- α production in our experiment was not dependent on stimulation with anti-IgE. Furthermore, we could detect TNF- α in the supernatants of basophil-depleted, but not basophil-enriched, preparations. We speculate that the source of TNF- α in our samples is likely to be monocytes, which are known to have receptors for IL-3^{21,22} and can express TNF- α mRNA.²³ Nevertheless, we cannot as yet clearly rule out that the TNF- α is in part produced by T cells or natural killer cells.

Although Cohen et al¹⁰ described that IL-3 can enhance cytokine production by LPS-stimulated macrophages, our studies do not support a role for LPS (as a contaminant) acting as the stimulus for TNF- α secretion. In our cultures with added polymyxin B, some reduction in TNF- α production was noted, but this was the case for both control samples and rhIL-3/S8- and rhIL-3/P8-stimulated samples, and thus the fold increase for both forms was not different.

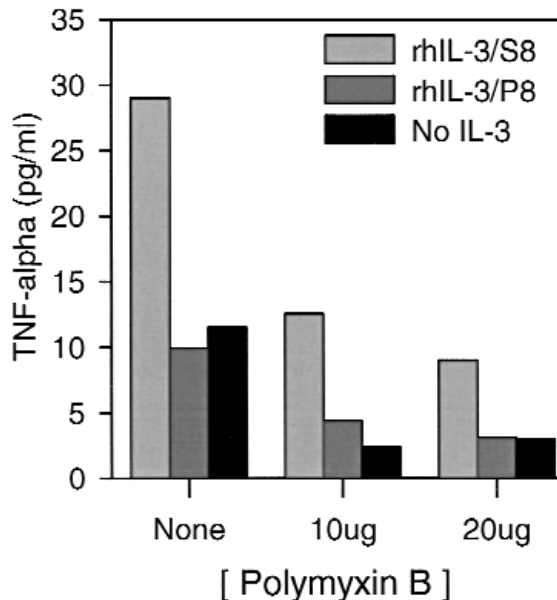


FIG 5. Polymyxin B does not inhibit the IL-3 enhancement over control of TNF- α secretion from human leukocytes. Leukocytes from one donor were stimulated with rhIL-3/S8 or rhIL-3/P8 at 10^{-8} mol/L in the presence or absence of polymyxin B at 10 or 20 μ g and incubated for 24 hours.

TNF- α is a pluripotent proinflammatory cytokine, and TNF- α and IL-4 are often regarded as having antagonistic functions. Nevertheless, both have been suggested to play important roles in the pathogenesis of the late response of bronchial asthma observed after antigen challenge.^{19,24} Thus our study suggests that further investigation is needed to determine whether differences in *IL3* genotype are associated with alterations in inflammatory, allergic, and/or other immune responses.

The results presented here suggest an effect on the relative therapeutic value of the 2 forms of IL-3. The rhIL-3/P8 form has significantly greater potency for inducing proliferation of a hematopoietic cell line, significantly less potency in priming basophils for LT and IL-4 release, and an undetectable capacity (over the concentration range tested) to induce TNF- α from peripheral blood cells. Thus these *in vitro* results would appear to translate to substantially reduced side effects for this form of IL-3 when used for enhancing bone marrow regeneration compared with the rhIL-3/S8 form. The concentrations used are equivalent to those reported in recent clinical trials.²⁵

In summary, we present data to demonstrate marked differences in 2 naturally occurring polymorphic forms of human IL-3. These 2 forms, which differ in a single amino acid at position 8 in the mature protein, were found to have reversed relative potencies in cells that depend on IL-3 for proliferation compared with activities related to inducing or priming for mediator release from basophils and other leukocytes.

Further studies are needed to establish the molecular basis providing the differences in function demonstrated here, to establish in vivo the effect on the therapeutic potential of these 2 forms of IL-3, and to determine any phenotypic differences associated with the endogenous production of these 2 different forms of IL-3.

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