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Phage Presentation and Affinity Selection of a Deletion Mutant of Human Interleukin-3

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ABSTRACT

A deletion derivative of the cytokine human interleukin-3 (hIL-3^{15-125}, comprising amino acids 15–125 of the native protein) was produced as a fusion to the filamentous phage surface protein pIII. The cytokine was detected in association with phage particles by protein immunoblotting. Compared to an equivalent quantity of soluble cytokine, phage-presented hIL-3^{15-125} exhibited reduced biological activity in a hIL-3-dependent cell proliferation assay. The reduction in activity was attributable to presence of phage particles in the assay, rather than directly owing to physical incorporation of the cytokine into the phage particle. Owing to the position of the amber codon in the phagemid vector, the phagemid-produced free hIL-3^{15-125} species (designated hIL-3^{15-125} ε) had 20 amino acids appended to its C-terminus; hIL-3^{15-125} ε did not exhibit reduced bioactivity. hIL-3^{15-125}-presenting phage were affinity-selected with either a hIL-3-reactive polyclonal antibody or with cells expressing the heterodimeric hIL-3 receptor. These data are consistent with the use of phage-display technology for the affinity selection of hIL-3 variants with modified biological properties.

Index Entries: Phage display; cytokine; receptor; growth factor; proliferation; biopanning.

Abbreviations: aa, amino acid; Ab, antibody; AML, acute myelogenous leukemia cell; Ap, ampicillin; BBS, borate buffered saline; BHK, baby ham-

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ster kidney cell; bp, base pair(s); BSA, bovine serum albumin; bST, bovine somatotropin (growth hormone); CAPS, 3-(cyclohexylamino)-1-propanesulphionic acid; Cb, carbenicillin; cpm, counts per minute; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; hCNTF, human ciliary neurotrophic factor; hG-CSF, human granulocyte colony-stimulating factor; hGH, human growth hormone; hGM-CSF, human granulocyte-macrophage colony-stimulating factor; hIL-3, human interleukin-3; hIL-3R, receptor for human interleukin-3; hIL-5, human interleukin-5; Km, kanamycin; pIII, tail protein of filamentous bacteriophage; PCR, DNA polymerase chain reaction; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; TU, titerable unit of phage; V, volts.

INTRODUCTION

The multilineage proliferation/differentiation activities of human interleukin-3 (hIL-3) *(1)* make it a candidate for the alleviation of cytopenia caused by myelosuppressive therapy *(2,3)* and for ex vivo expansion of blood progenitor cells *(4,5)*. The biological activities of hIL-3 are mediated by specific ligand–receptor interaction. The receptor complex contains a hIL-3-specific α subunit, capable of low-affinity ligand binding, and a β_c subunit, common to human granulocyte colony stimulating factor (hGM-CSF) and human interleukin-5 (hIL-5) receptor complexes *(6)*. Both subunits are needed for high-affinity binding and, as far as is known, for signal transduction.

Previously, hIL-3 was produced in a polyvalent phage display system for use in anti-hIL-3 antibody production *(7)*. The putatively phage-presented hIL-3 was potently immunogenic and modestly active in a cell-proliferation assay, but direct demonstration of physical association between hIL-3 and phage particles and affinity selection of presenting phage were not reported.

Use of phage presentation to augment the immunogenicity of the presented protein is an atypical application of display technology. More commonly, display methods are used to affinity-select variants from a library of displayed proteins or peptides. In this application, the technique utilizes linkage of the displayed protein/peptide to the genetic element that encodes it *(8,9)*. Some hIL-3 variants may owe their enhanced receptor–agonist activity at least in part to increased affinity for the receptor *(10)*, suggesting that affinity selection, used to screen display libraries (i.e., biopanning), may be appropriate for identification of hIL-3 molecules with enhanced biological properties. In order to establish the feasibility of phage display of hIL-3 for affinity selection of variant molecules, a selected, genetically engineered mutant of the cytokine was produced by a

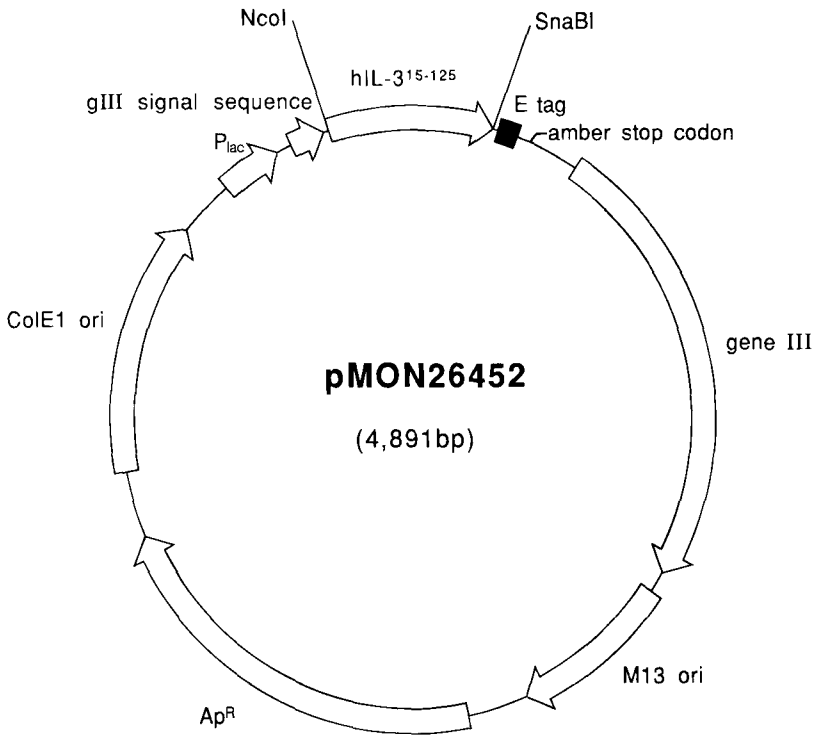

Fig. 1. hIL-3^{15-125} was produced using pMON26452, a phagemid vector for monovalent presentation. Selected genetic elements of the phagemid are indicated. pMON26452 produces a fusion protein comprising (from the amino to carboxy termini) the M13 pIII signal peptide, hIL-3^{15-125}, the E tag peptide, and the main body of the pIII tail protein.

monovalent phagemid system. Association of this hIL-3 molecule with phage particles was demonstrated. Cytokine-presenting phage were preferentially affinity selected with each antibody to hIL-3 and with cells presenting the heterodimeric hIL-3 receptor on their surfaces.

MATERIALS AND METHODS

Construction of Phagemid Vector

The pCANTAB 5 E phage-display vector (Pharmacia Biotech) was modified by standard molecular biology methods *(11)* to produce pMON26452 (Fig. 1). A synthetic oligonucleotide (5′-CGGCCATG-GCATAGTAGTAACGCATTCTACGTAGC-3′) containing *Nco*I and *Sna*BI restriction sites was inserted between the *Sfi*I and *Not*I sites of pCANTAB 5 E to produce pMON26444. The unique *Eco*RI site at the 3′ end of gene *III* was eliminated from *Eco*RI-digested pMON26444 using the Klenow fragment of DNA polymerase I to fill in the staggered ends of the cleaved

restriction site, producing pMON26445. An *Nco*I-*Sna*BI DNA fragment comprising the coding sequence for hIL-3^{15-125} *(12)*, plus an additional codon (TAC, specifying tyrosine, and forming half of a *Sna*BI restriction site) fused in-frame at the end of the coding sequence, was isolated. This fragment was inserted into the *Nco*I-*Sna*BI sites of pMON26445 to generate pMON26452. A second plasmid, pMON26446, analogous to pMON24645, but containing a bovine somatotropin (bST) coding sequence between its *Nco*I and *Sna*BI sites, was made for use as a negative control that presents a four helical bundle protein (bST) that does not recognize the hIL-3 receptor.

Phage

Phage were propagated by growth in 2^{x}YT liquid culture *(11)*, with appropriate antibiotic, following co-infection of *Escherichia coli* strain JM101 (Stratagene) with helper phage M13KO7 (Promega) and phage derived from either pMON26452 or pMON26446. Concentrated phage were obtained from saturated cultures by polyethylene glycol (PEG) precipitation: a cleared supernatant of a saturated culture was prepared by centrifugation at 17,700*g* for 20 min at 4°C; 0.15 vol PEG solution (16.7% [w/v] PEG [Sigma], average MW 8,000, 3.3 *M* NaCl) was added and after thorough mixing placed on ice at least 3 h; phage were pelleted by centrifugation at 12,800*g* for 30 min at 25°C, then resuspended in Dulbecco's phosphate buffered saline (DPBS; GIBCO/BRL). Phage for use in affinity selection (biopanning) experiments were subjected to a second round of percipitation with PEG and resuspended in DPBS with 1% (w/v) BSA (Sigma) and 1% (w/v) sodium azide (Fisher Scientific). Instead of the second PEG percipitation step, phage used in cell proliferation experiments were purified by isopycnic banding from a solution of 1.3 g CsCl (Sigma) per mL DPBS centrifuged at 85,000*g* in a VTi50 rotor (Beckman) for at least 20 h at 25°C, followed by pelleting by centrifugation at 85,000*g* in a 45Ti rotor (Beckman) for 4 h at 25°C, and resuspension in DPBS. Titerable units (TU) of phage were determined by titering on JM101. Cells were grown to density corresponding to Klett 100 (Clinical Model Photoelectric Colorimeter with green filter; Manostat), pelleted by low-speed centrifugation, starved by incubation in 80 m*M* NaCl for 1 h, with gentle shaking, at 37°C, repelleted and resuspended in 1 mL super optimal catabolite (SOC) medium (GIBCO/BRL) per 25 mL starting culture. Cells were infected with 10^{-4} dilution in DPBS of phage stock, incubated stationary 10 min at room temperature, then 30 min with slow shaking at 37°C, and dilutions in LB *(11)* plated on LB agar with either kanamycin or carbenicillin at 75 μg/mL, followed by incubation at 37°C and counting of antibiotic-resistant colonies. Concentrations of phage particles were determined as described *(13)* using a Beckman DU-40 Spectrophotometer.

Elisa

Phage samples were evaluated using a sandwich ELISA that detects hIL-3, as described previously *(12)*. The lower limit of sensitivity for detection of hIL-3 was 6.7×10^{-2} pmol/mL.

Western Blot Analysis

10^{11} TU per lane of virions were electrophoresed through duplicate nondenaturing agarose gels *(14)*. Following electrophoresis, each gel was transferred to Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) by electroblotting at 100 V for 90 min at 4°C in a buffer consisting of 10 m*M* CAPS (Sigma), pH 11.0, 10% methanol, and 0.005% SDS. One gel was stained with Coomassie brilliant blue *(15)*, whereas the other was subjected to Western-blot analysis *(11)*. This blot was stained with antibody to a hIL-3 peptide *(12)* and staining was detected using a secondary antibody conjugated to horseradish peroxidase (Amersham).

Production of Soluble Cytokine and Cell Proliferation Assay

hIL-3^{1-133} was purified and the tritiated thymidine incorporation hIL-3-dependent AML193.1.3 cell proliferation assay was performed as described *(12)*. The polyclonal antibody to hIL-3, used in control experiments to abolish proliferative activity, has been previously described *(12)*. CsCl gradient-purified phage were prepared, quantitated, and the hIL-3^{15-125} concentrations in the phage preparations were determined by ELISA, as previously described. hIL-3^{15-125} ε was produced using the *E. coli* strain W3110 *(16)* by osmotic shock as described *(12)*. Soluble hIL-3 species were quantitated by ELISA.

Affinity Selection

Phage for affinity-selection experiments were concentrated by two successive PEG precipitations and resuspended in DPBS with 1% (w/v) BSA (Sigma) and 1% (w/v) sodium azide (Fisher Scientific). A 50 µL input mixture comprising pMON26452-derived phage and nonpresenting M13KO7 phage was incubated with the selective agent in binding buffer (DPBS, including Ca, Mg, with 1% (w/v) BSA). Affinity selection was performed with either of two different selective agents: *(1)* Wells of a microtiter plate (Maxisorp Immuno Plate, Nunc) were coated overnight at 4°C with 50 µL of a 10 µg/mL solution in borate buffered saline (6.18 g boric acid, 9.5 g sodium borate, 8.8 g sodium chloride, each per L; pH 8.2) of either goat polyclonal anti-SC55494 (a recombinant hIL-3 derivative [*10*]), which cross-reacts with hIL-3^{15-125}, or rabbit polyclonal anti-bST *(17)*. After decanting, wells were blocked with 200 µL DPBS with 1% (w/v) BSA for 2 h at room temperature.

Blocking solution was removed and phage-input mixture was added, followed by incubation overnight at 4°C. Following aspiration of supernatant, washes and elution were performed as described below, with the exception that plates were not centrifuged; *(2)* Baby-hamster kidney (BHK) cells stably expressing the herpes virus transcriptional transactivating protein VP16 *(18)* were cotransfected with a plasmid expressing the AML193.1.3 cell-derived hIL-3Rα gene *(10)* from the VP16-responsive promoter of the herpes virus IE175 gene and a plasmid conferring resistance to hygromycin. A hygromycin-resistant clone that demonstrated specific binding by ^{125}I-hIL-3 *(10)* was retransfected with a plasmid expressing the hIL-3Rβ gene (obtained by reverse transcription-polymerase chain reaction (RTPCR) from human eosinophils) from the IE175 promoter and with a plasmid conferring resistance to histidinol. That histodinol-resistant clone (BHK/IL-3R) expressing the highest affinity binding to the genetically engineered hIL-3 derivative SC55494 *(10)* was used as a selective agent for affinity-selection of phage. BHK/IL-3R cells were maintained in 7% CO_2 in DMEM supplemented with 5% (v/v) FBS and 2 m*M* L-glutamine (each from GIBCO/BRL), harvested by treatment with Nonenzymatic Cell Dissociation Solution (Sigma), washed with and resuspended at 3×10^7/mL in binding buffer. Affinity-selection was performed with 200 µL cell suspension in a 6-mL tube, with gentle mixing for 4 h at room temperature and then overnight at 4°C. Following centrifugation and aspiration of supernatant, pellets containing bound phage were washed with gentle mixing at room temperature in DPBS, without Ca, Mg, with 0.2% (w/v) BSA, for various lengths and number of washes. Following the final wash, samples were pelleted by low-speed centrifugation and wash buffer aspirated prior to elution of bound phage. Each sample was then resuspended in elution buffer (0.2 *M* HCl-glycine, pH 2.2, with 0.1% [w/v] BSA) and incubated stationary for 30 min at room temperature. After centrifugation, supernatant was decanted and neutralized by addition of 0.075 vol 2 *M* Tris-HCl, pH 9.5. Competence of BHK/IL-3R cells for binding hIL-3 was demonstrated by incubation of cell suspension with ^{125}I-hIL-3^{15-125} (with an additional C-terminal tyrosine residue to facilitate efficient labeling with ^{125}I) overnight at 4°C, and comparison of ^{125}I in centrifuged cell pellet to total, added cpm. The number of high-affinity (hIL-3R$\alpha\beta_c$) binding sites/cell was estimated to be $1.4 \pm 0.04 \times 10^4$.

RESULTS AND DISCUSSION

Construction of a Phagemid Vector for Presentation of a Deletion Mutant of hIL-3 on Virions

Previously, full-length hIL-3 was presented in multiple copies on filamentous bacteriophage *(7)*. Because the objective of that study was not

affinity-selection of hIL-3-phage, but rather the production of hIL-3-phage for use as an immunogen, the presentation strategy was appropriate. Such polyvalent presentation, however, can potentially result in "chelate" effects, whereby phage-presenting proteins with individually low affinities exhibit high overall avidity for target molecules, owing to the display of multiple copies of the protein *(8)*. In affinity selection procedures, chelate effects may result in diminished discrimination among variants with different binding properties. In some cases, this concern might be addressed by reducing the concentration of the target molecules to a level that makes it unlikely that any given phage would interact with multiple target molecules. Because the hIL-3 receptor is a multi-protein complex that has not been demonstrated to form in solution, production of an affinity reagent with both a low concentration of receptor complexes and with the preponderance of the receptor subunits incorporated into complexes is technically difficult. Consequently, hIL-3^{15-125} was produced using a phagemid vector in order to achieve "monovalent" presentation *(8)*.

The phagemid pMON26452 is shown in Fig. 1. It is derived from pCANTAB 5 E (Pharmacia Biotech) and directs the synthesis of a fusion protein comprising amino acids 15 through 125 of hIL-3 (hIL-3^{15-125}), a peptide tag (E tag), followed by the main body of the filamentous phage pIII tail protein. The deletion mutant hIL-3^{15-125}, rather than the full-length cytokine, was presented because it is efficiently secreted into the periplasm of *E. coli* and is modestly more active in cell-proliferation assays than is the larger hIL-3^{1-133} species *(12)*. In the presence of helper phage M13KO7 *(19)*, single-stranded DNA from pMON26452 can be packaged into virions.

Characterization of Phage Produced from Phagemid Vector

Enzyme-linked immunosorbent assay (ELISA) measurement showed that there was 2.3 pmol/mL of immunoreactive hIL-3 in a cesium chloride gradient-purified preparation of virions derived from pMON26452. This preparation contained 8×10^{11} TU/mL, as measured by titering on *E. coli* JM101 cells. Based on spectrophotometric quantitation, there were 1.1×10^{14} physical-phage particles per mL in this preparation. Thus, the preparation contained approx 1.3 hIL-3^{15-125} molecules per 100 virions. This result is consistent with results reported for monovalent presentation of hGH, for which it was estimated that 10% of phage particles contained one hGH-pIII fusion protein *(20)*, and hCNTF, for which it was reported that 1.5% of phage presented hCNTF *(21)*. As a negative control, each of these measurements was performed on a phage preparation produced from the pCANTAB 5 E-derived presentation vector pMON26446. These pMON26446-derived phage displayed a nonprimate, four-helical bundle protein in place of hIL-3^{15-125} (bST), but were otherwise identical to pMON26452-derived

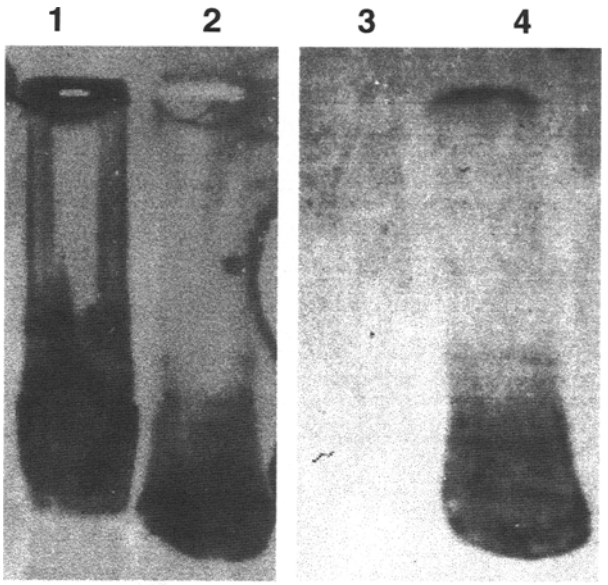

Fig. 2. Western-blot analysis of intact phage. Lanes 1 and 3 contain virions of the helper phage M13KO7 and lanes 2 and 4 contain virions made from the phagemid construct pMON26452. Lanes 1 and 2 are stained with Coomassie brilliant blue; lanes 3 and 4 are stained with antibody to hIL-3.

phage. The cesium chloride gradient-purified preparation of pMON26446-derived phage contained 3×10^{11} TU/mL and 8.4×10^{13} physical particles per mL. There was no detectable immunoreactive hIL-3 in pMON26446-derived phage preparations.

ELISA detection of hIL-3 in phage preparations did not conclusively establish that the cytokine was incorporated into virions. Previous attempts to present hIL-3^{15-125} on phage failed as the result of inadvertent introduction of a protease cleavage site into a linker between the cytokine and pIII (S. C. Lee, unpublished results). Consequently, even though antibody-detectable hIL-3 was present in CsCl gradient-purified phage preparations, little, if any, was incorporated into virions. Additionally, because the efficiency of suppression of an amber codon (present in pMON26452 between coding sequences for hIL-3^{15-125} and pIII) was expected to be modest *(22)*, hIL-3 detected using the ELISA might have been distributed between the phage-associated fusion protein and the free, soluble form. To determine whether hIL-3^{15-125} had been incorporated into virions, intact particles were electrophoresed, transferred to a membrane and stained with antibody to hIL-3 (Fig. 2). Because the single-stranded form of pMON26452 DNA that was packaged into virions was smaller than the corresponding DNA of M13KO7, the assembled pMON26452-phage were

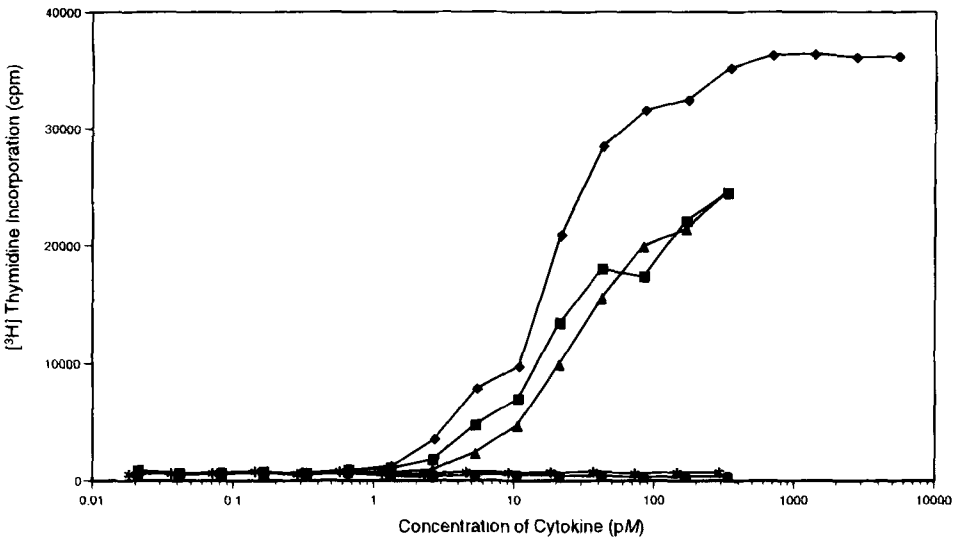

Fig. 3. Activities of pMON26452-derived phage and of soluble hIL-3 in a tritiated thymidine incorporation, hIL-3-dependent, cell proliferation assay. Filled diamonds (◆) indicate soluble hIL-3^{1-133} Asterisks (*) indicate hIL-3^{1-133} plus a polyclonal antibody directed to hIL-3. Filled squares (■) indicate the pMON26452 (hIL-3^{15-125} presenting) phage preparation. Filled circles (●) indicate pMON26452 phage preparation plus anti-hIL-3. Filled triangles (▲) indicate pMON26446 (non-hIl-3^{15-125}-presenting; see Materials and Methods section) phage preparation with exogenously added hIL-3^{1-133}. Quantities of soluble hIL-3^{1-133} used to supplement the pMON26446 preparation were matched to the amount of hIL-3^{15-125} detected by ELISA in the pMON26452 phage preparation. For each data point, the number of pMON26446-derived phage particles was equal to that of pMON26452-derived phage particles. Data are the mean of two determinations.

smaller than these nonpresenting helper phage and consequently migrated farther.

Coomassie staining of a duplicate membrane showed that comparable amounts of the helper phage and pMON26452-phage (Fig. 2, Lanes 1 and 2, respectively) were loaded. pMON26452-phage stained with antibody to hIL-3 (Fig. 2, Lane 4), whereas M13KO7 helper phage, produced in the absence of pMON26452, did not (Fig. 2, Lane 3), consistent with assembly into virions of hIL-3^{15-125} produced from pMON26452 as a fusion to pIII. In order to further address the question of whether the detected hIL-3^{15-125} was specifically associated with phage particles, a control experiment was performed in which a mixture of nonpresenting M13KO7 phage and soluble hIL-3^{15-125} was subjected to an identical Western analysis. No detectable staining with anti-hIL-3 was observed in this case (data not shown). This result eliminates the hypothesis that the antibody-detectable hIL-3 in the pMON26452-phage preparations was nonspecifically trapped by the phage particles. Taken

together, the results of the Western analysis are consistent with incorporation of the hIL-3^{15-125} into the pMON26452-derived phage particles.

Biological Activity in pMON26452-phage Preparations

pMON26452 phage preparations stimulated cell proliferation (Fig. 3) in a hIL-3-dependent, AML193.1.3 cell proliferation assay *(12)*. Cesium chloride density gradient purification was required to obtain bioactive material (virions prepared by two, consecutive PEG precipitations, as described in the Materials and Methods Section, but without density gradient fractionation, were not active in the proliferation assay; data not shown). The proliferative activity associated with the pMON26452-phage preparations was owing to the presence of hIL-3: the addition of neutralizing, polyclonal antibody to hIL-3 abolished proliferation. Addition of the same concentration of a polyclonal antibody to hG-CSF did not reduce the proliferative response to hIL-3 (data not shown), demonstrating that the effect of the anti-hIL-3 antibody on cell proliferation was specific. Furthermore, a cesium chloride density gradient-purified preparation of non-hIL-3-presenting, negative-control phage produced from pMON26446 did not stimulate detectable AML193.1.3 proliferation (data not shown).

The proliferative response induced by hIL-3^{15-125}-phage preparations was diminished relative to that induced by preparations of soluble hIL-3^{1-133} produced from the expression vector pMON5873 *(12)*. This hIL-3 species, which was available in a sufficient quantity for assay, has been previously shown to have a modestly reduced activity relative to hIL-3^{15-125} *(12)*.

Virions themselves mediated an inhibitory effect on hIL-3-driven proliferation of AML193.1.3 cells. When a preparation of the non-hIL-3-presenting, negative-control phage produced from pMON26446, containing the same number of virions as the preparation of pMON26452-derived particles, was supplemented with an amount of hIL-3 equivalent to that detected in the pMON26452 preparation by ELISA, this hIL-3-supplemented pMON26446-phage preparation showed a reduction in the proliferative response comparable to that exhibited by the pMON26452-phage preparation (Fig. 3). The data are consistent with the hypothesis that the presence of the phage particles in the assay mediated the reduction in proliferative response observed with the cytokine-presenting pMON26452 preparation.

Reductions in bioactivity of phage-presented molecules have been reported previously. The activity of hIL-3 was dramatically reduced when the cytokine was presented in a polyvalent phage display format *(7)*. The reduction in bioactivity was substantially greater than that shown in Fig. 3 for monovalently presented hIL-3^{15-125}. In another case, the bioactivity of the growth factor hCNTF was modestly diminished when presented on phage *(21)*. Differences among the three results may be attributable to

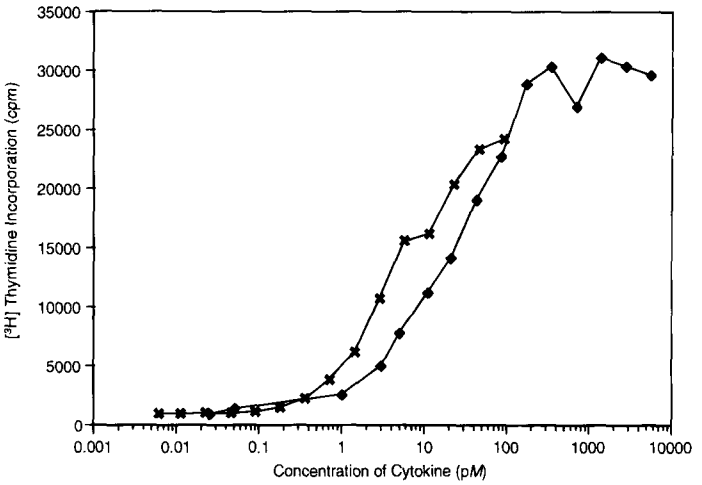

Fig. 4. Activity of the soluble, modified hIL-3^{15-125} (designated hIL-$3^{15-125}\varepsilon$) protein produced from pMON26452 in a tritiated thymidine incorporation, hIL-3-dependent, cell-proliferation assay. Filled diamonds (◆) indicate purified hIL-3^{1-133}. Crosses (✖) indicate hIL-$3^{1-125}\varepsilon$. Data are the mean of two determinations.

differences in presentation (monovalent vs polyvalent; differences in the position of the fusion to pIII), biological differences among the presented growth factors, or differences in the sensitivity of the respective assay systems to the potentially inhibitory effect of phage particles.

Biological Activity of Soluble hIL-3 Species Produced from pMON26452

A soluble species of hIL-3 was prepared from pMON26452 by osmotic shock in the nonsuppressing *E. coli* host W3110 *(16)*, so that protein synthesis terminated at the amber codon between the hIL-3^{15-125} and pIII moieties (Fig. 1). As a consequence of the way in which pMON26452 was constructed, the amino-acid sequence Tyr-Val-Ala-Ala-Ala-Gly-Ala-Pro-Val-Pro-Tyr-Pro-Asp-Pro-Leu-Glu-Pro-Arg-Ala-Ala was appended to the C-terminus of hIL-3^{15-125} produced from pMON26452 in *E. coli* W3110; this soluble species was designated hIL-$3^{15-125}\varepsilon$. The data presented in Fig. 4 show that hIL-$3^{15-125}\varepsilon$ exhibited proliferative activity comparable to that previously reported for hIL-3^{15-125} *(12)*. It was slightly more potent than hIL-3^{1-133} and did not exhibit the diminished proliferative activity observed with pMON26452-phage preparations (Fig. 3). Negative control preparations from osmotic shocks of a culture of W3110 that did not contain pMON26452 exhibited no detectable proliferative activity in the AML193.1.3 assay (data not shown).

As described in the previous section, the phage-displayed form of hIL-3^{15-125} was active in cell-proliferation assays, but was subject to partial inhibition of activity owing to presence of phage particles in the assay. This result is consistent with use of phage-displayed growth factors in cell-proliferation assays to determine relative potencies, but such assays are less likely to yield data that accurately correlate with the activity of the soluble form of the factor. Considering both the observed "virion interference" effect in the cell-proliferation assay and the necessity for laborious purification of phage preparations using cesium chloride density gradients prior to bioassay, direct assay of hIL-3^{15-125} in phage preparations was inconvenient, implying that this approach will be less practical than assay of variant factors expressed in soluble form.

Affinity Selection of pMON26452-Derived Phage

Because Western-blot analysis was consistent with the incorporation of hIL-3^{15-125} into virions (Fig. 2), a hIL-3-reactive polyclonal antibody (described in the Materials and Methods Section) was used as a reagent to select putative hIL-3^{15-125}-presenting phage particles in biopanning experiments. As described in the Materials and Methods Section, an input mixture comprising Cb^R-conferring, putative hIL-3^{15-125}-presenting pMON26452-phage and a large excess of $K_m{}^R$-conferring, non-hIL-3^{15-125}-presenting M13KO7 negative-control phage was applied to a microtiter well-coated with either anti-hIL-3 or anti-bST. After an interval allowing interaction of phage with selective agent, the wells were washed to remove unbound phage, and bound phage were eluted and titered on *E. coli*. Comparison, in Table 1, of Cb^R TU/mL obtained by selection on anti-hIL-3 with Cb^R TU/ml obtained by selection on anti-bST shows that pMON26452 particles were vastly more efficiently selected using hIL-3-reactive antibody than with the negative control antibody to bST. The relative enrichment of pMON26452-phage compared to M13KO7, owing to affinity selection, is calculated as the ratio of Cb^R TU/mL to Km^R TU/mL in the eluted output divided by that same ratio in the input mixture; by definition, the enrichment for the input mixture is equal to 1. When anti-hIL-3 was the selective agent, pMON26452-phage were enriched 6900-fold compared to M13KO7. These results are consistent with the presentation of hIL-3^{15-125} in a properly folded conformation on a subset of pMON26452-derived phage, as expected given the amount of hIL-3^{15-125} per phage particle measured by ELISA.

Although pMON26452 particles were efficiently affinity selected using antibody as selective agent, experience with displayed peptides selected using antibodies as a substitute for cognate target proteins suggests that antibody may not be the optimal selective agent for screening display

Table 1
Affinity-Selection of pMON26452-Derived Phage

Sample	Selective agent	Km^R TU/mL	Cb^RTU/mL	Ratio $\left(\frac{Cb^R TU/mL}{K_m{}^R/TU/mL}\right)$	Enrichment
Input mixture	(not applicable)	1.3×10^{13}	1.4×10^{11}	1.1×10^{-2}	1
Eluate following:					
6 × 60 min wash	anti-IL-3 Ab	5.8×10^{7}	4.4×10^{9}	76	6,900
6 × 60 min wash	anti-bST Ab	5.4×10^{8}	4.4×10^{7}	8.1×10^{-2}	7.4
Input mixture	(not applicable)	4.3×10^{13}	2.2×10^{11}	5.0×10^{-3}	1
Eluate following:					
1 × 5 min wash	BHK/IL-3R	2.1×10^{6}	1.1×10^{6}	5.2×10^{-1}	104
	BHK	5.1×10^{5}	2.9×10^{3}	5.7×10^{-3}	1.1
1 × 30 min wash	BHK/IL-3R	1.0×10^{6}	4.4×10^{5}	4.4×10^{-1}	88
	BHK	2.2×10^{5}	1.9×10^{3}	8.6×10^{-3}	1.7
3 × 30 min wash	BHK/IL-3R	5.2×10^{5}	4.2×10^{5}	8.1×10^{-1}	162
	BHK	1.4×10^{4}	4.0×10^{2}	2.9×10^{-2}	5.7
5 × 30 min wash	BHK/IL-3R	5.7×10^{4}	2.5×10^{5}	4.4	880
	BHK	5.3×10^{3}	1.8×10^{2}	3.4×10^{-2}	6.8

libraries to select protein variants with enhanced activities. For example, peptides identified using anti-biotin antibodies as affinity reagent exhibited poor binding to the authentic cognate target protein, streptavidin *(23)*. Because the hIL-3R is a heterodimer, solid supports, such as agarose or synthetic beads presenting either or both hIL-3 receptor subunits, were also rejected as affinity reagents. To perform selection with a reagent that presented the hIL-3 receptor in a presumably physiologically authentic context, mammalian cells that present both hIL-3 receptor subunits on their surfaces, and which are known to allow the formation of high-affinity, oligomeric hIL-3R complexes (J. Thomas, personal communication), were used for affinity selection of pMON26452 particles. Flow cytometric quantitation using monoclonal antibodies specific for each of the hIL-3 receptor subunits indicated the α and β_c subunits to be present on the surfaces of BHK/IL-3R cells used in the biopanning experiments at a ratio of about two α subunits to one β_c subunit (L. E. Kahn, personal communication). These hIL-3R-presenting cells allowed the preferential affinity selection of pMON26452 phage particles from an input mixture containing a large excess of nonpresenting M13KO7 phage, as shown in Table 1.

Whole cells have previously been used as affinity selection agents in phage-display biopanning experiments *(24,25)*. Because each of these studies used a phage-displayed peptide library, it was not *a priori* evident that a larger, phage-displayed protein would be properly folded and available to interact with a cell-presented receptor, such that it could be specifically affinity selected. Thus, the results reported here extend the earlier uses of cells as affinity selection agents by demonstrating that a 111 amino acid protein could be presented on phage and preferentially selected using receptor-presenting cells. Additionally, whereas the cells in the previous studies were not engineered to express a heterologous surface molecule, the results of the present work demonstrated the application of a heteromeric receptor, expressed in a heterologous cell type, as an affinity selection agent.

The data in Table 1 show that increasing enrichment of pMON26452-phage selected with BHK/IL-3R cells was obtained with increasing number of washes. The outcome of this representative experiment was reproduced in several experiments, although the point at which a large increase in enrichment occurred varied between 3 and 5 washes (data not shown). The data in Table 1 from multiple 30 min washes are consistent with a preferential elimination of nonpresenting M13KO7 with extensive washing of BHK/IL-3R cells, concomitant with retention of a nearly constant amount of hIL-3-presenting phage (compare Km^R TU/mL to Cb^R TU/mL). This result is promising with respect to the use of this system to affinity select molecules from a library of variants, because in that case it is desirable to wash away those molecules that exhibit a lower affinity for the selective agent. Considering that some known hIL-3 variants may owe their

enhanced receptor-agonist activity at least in part to their increased affinity for the receptor *(10)*, the data presented here support the notion that phage display of hIL-3^{15-125} variants and affinity-selection with the cell-presented, heterodimeric, high-affinity hIL-3R complex is a feasible approach for the identification of molecules with enhanced biological activity.

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REFERENCES

1. Metcalf, D. (1993), *Blood* **82,** 3515–3523.
2. Biesma, B., Willemse, P. H. B., Mulder, N. H., Sleijfer, D. Th., Gietema, J. A., Mull, R., Limburg, P, C., Bouma, J., Vellenga, E., and de Vries, E. G. E. (1992). *Blood* **80,** 1141–1148.
3. Gianni, A. M., Siena, S., Bregni, M., Di Nicola, M., Peccatori, F., Magni, M., Ravagnani, F., Sklenar, L., and Banadonna, G. (1993). *Annals Oncol.* **4,** 759–766.
4. Zimmerman, T. M., Williams, S. F., Bender, J. G., Lee, W. J., Blake, M., Carreon, J., Swinney, P., Smith, S. J., Schilling, M., Oldham, F., Van Epps, D., and Williams, D. E. (1995), *J. Hematotherapy* **4,** 527–529.
5. Purdy, M. H., Hogan, C. J., Hami, L., McNeice, I., Franklin, W., Jones, R. B., Bearman, S. I., Berenson, R. J., Cagnoni, P. J., Heimfeld, S., and Shpall, E. J. (1995), *J. Hematotherapy* **4,** 515–525.
6. Miyajima, A., Mui, A. L.-F., Ogorochi. T., and Kazuhiro, S. (1993). *Blood* **82,** 1960–1974.
7. Gram, H., Strittmatter, U., Lorenz, M., Glueck, D., and Zenke, G. (1993), *J. Immunol. Meth.* **161,** 169–176.
8. Clackson, T. and Wells, J. A. (1994), *Tibtech* **12,** 173–184.
9. Winter, J. (1994), *Drug Devel. Res.* **33,** 71–89.
10. Thomas, J. W., Baum, C. M., Hood, W. F., Klein, B., Monahan, J. B., Paik, K., Staten, N., Abrams, M., and McKearn, J. P. (1995). *Proc. Natl. Acad. Sci.* **92,** 3779–3783.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
12. Olins, P. O., Bauer, S. C., Bradford-Goldberg, S., Sterbenz, K., Polazzi, J. O., Caparon, M. H., Klein, B. K., Easton, A. M., Paik, K., Klover, J. A., Thiele, B. R., and McKearn, J. P. (1995), *J. Biol. Chem.* **270,** 23,754–23,760.
13. Parmley, S. F. and Smith, G. P. (1988), *Gene* **73,** 305–318.

14. Boeke, J. D., Russel, M., and Model, P. (1980). *J. Mol. Biol.* **144,** 103–116.
15. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
16. Bachmann, B. J. (1987), in Escherichia coli *and* Salmonella typhimurium *Cellular and Molecular Biology,* Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds., American Society for Microbiology, Washington, D. C., pp. 1190–1219.
17. Wood, D. C., Salsgiver, W. J., Kasser, T. R., Lange, G. W., Rowold, E., Violand, B. N., Johnson, A., Leimgruber, R. M., Parr, G. R., Siegel, N. R., Kimack, N. M., Smith, C., Zobel, J. F., Ganguli, S. M., Garbow, J. R., Bild, G., and Krivi, G. G. (1989), *J. Biol. Chem.* **264,** 14,741–14,747.
18. Hippenmeyer, P. J. and Pegg, L. E. (1995), *Curr. Opin. Biotech.* **6,** 548–552.
19. Vieira, J. and Messing, J. (1987), *Meth. Enzymol.* **153,** 3–11.
20. Bass, S., Greene, R., and Wells, J. A. (1990), *Proteins: Structure, Function and Genetics* **8,** 309–314.
21. Saggio, I., Gloaguen, I., and Laufer, R. (1995), *Gene* **152,** 35–39.
22. Stent, G. S. and Calendar, R. (1978), *Molecular Genetics,* 2nd ed., Freeman, San Francisco.
23. Roberts, D., Guegler, K., and Winter, J. (1993), *Gene* **128,** 67–69.
24. Doorbar, J. and Winter, G. (1994), *J. Mol. Biol.* **244,** 361–369.
25. Barry, M. A., Dower, W. J., and Johnston, S. A. (1996), *Nature Medicine* **2,** 299–305.