

INTERLEUKIN-5 (IL-5) is a key mediator of eosinophilic inflammation. The biological role of this cytokine in an allergic airway inflammatory response has been widely demonstrated in guinea pigs, yet the interaction of guinea pig IL-5 (gpIL-5) with its receptor has not been studied. Experiments were performed to quantitate the interaction of gpIL-5 with gpIL-5r and to compare this affinity with that of hIL-5 and mIL-5 and their cognate receptors. The cross-species affinity and agonist efficacy were evaluated to see if gpIL-5r had a restricted species reactivity (as is the case with mIL-5r) or did not distinguish between IL-5 orthologs (similar to hIL-5r). gpIL-5 was cloned using mRNA isolated from cells obtained by bronchoalveolar lavage. Recombinant gpIL-5 was expressed in *T.ni* insect cells and purified from spent media. Binding assays were performed using insect cells expressing hIL-5r $\alpha\beta$ or gpIL-5r $\alpha\beta$ 1 as previously described (*Cytokine*, 12:858–866, 2000) or using B13 cells which express mIL-5r. The agonist potency and efficacy properties of each IL-5 ortholog were evaluated by quantitating the proliferative response of human TF-1 cells and murine B13 cells. gpIL-5 bound with high affinity to recombinant gpIL-5r as demonstrated by displacing [¹²⁵I]hIL-5 ($K_i = 160$ pM). gpIL-5 also bound to hIL-5r with high affinity ($K_i = 750$ pM). hIL-5 and mIL-5 showed similar, high-affinity binding profiles to both gpIL-5r and hIL-5r. In contrast, gpIL-5 and hIL-5 did not bind to the mIL-5r as demonstrated by an inability to displace [¹²⁵I]mIL-5, even at 1000-fold molar excess. These differences in affinity for IL-5r orthologs correlated with bioassay results: human TF-1 cells showed roughly comparable proliferative responses to guinea pig, human and murine IL-5 whereas murine B13 cells showed a strong preference for murine over guinea pig and human IL-5 ($EC_{50} = 1.9, 2200$ and 720 pM, respectively). Recombinant gpIL-5 binds to the gpIL-5r with high affinity, similar to that seen with the human ligand-receptor pair. gpIL-5r and hIL-5r do not distinguish between the three IL-5 orthologs whereas mIL-5r has restricted specificity for its cognate ligand.

Key words: Eosinophil, Interleukin-5, Asthma, Airway inflammation

Expression and *in vitro* properties of guinea pig IL-5: Comparison to human and murine orthologs

Clay W Scott^{CA}, Carol Budzilowicz,
Stephen J. Hubbs, Mark Stein,
Cindy Sobotka-Briner and Deidre E. Wilkins

AstraZeneca Pharmaceuticals, CNS Discovery
Research, LW208, 1800 Concord Pike, Wilmington,
DE 19810, USA

^{CA} Corresponding Author

Tel: + 1302-886-3177

Fax: + 1302-886-4803

E-mail: Clay.Scott@AstraZeneca.com

Introduction

Activated eosinophils can produce a wide array of pro-inflammatory mediators and bronchoconstricting agents (reviewed in refs^{1,2}). The increased numbers of eosinophils seen in human allergic airways^{3,4} and the elevated levels of eosinophil products measured in bronchoalveolar lavage (BAL) from such patients^{3,5} support the hypothesis that eosinophilic inflammation is an important pathogenic component of chronic asthma. IL-5 is important for eosinophil development, mobilization from bone marrow, and activation in the allergic airway. Characterization of

this cytokine and an understanding of its role has led to various therapeutic approaches to block its production and or actions (see refs⁶⁻⁸ for reviews).

Guinea pigs have proven a valuable species for simulating certain aspects of airway inflammation. For example, both allergen and IL-5 induced release of eosinophils from guinea pig bone marrow and their accumulation in airways have been demonstrated.⁹⁻¹¹ Furthermore, both *in vivo* and *in vitro* studies are commonly performed with guinea pigs to evaluate therapeutic modalities aimed at reducing the allergic response in humans. Although numerous studies have described the molecular characteristics

of human and murine IL-5 and their interaction with cognate receptors,¹²⁻¹⁶ little is known about the guinea pig ligand/receptor system. Guinea pig IL-5 (gpIL-5) has been cloned, expressed and shown to induce airway eosinophilia in guinea pigs when given by tracheal injection.^{11,17} We recently reported the cloning and expression of guinea pig IL-5 receptor α subunit and multiple β subunits.¹⁸ We also reconstituted a high affinity gpIL-5 $\alpha\beta_1$ complex using hIL-5 as ligand. In this report, we extend these studies to show the high affinity interaction of guinea pig IL-5 with its recombinant receptor. We also demonstrate its cross-species affinity and agonist activity with the human and murine IL-5r and compare these results with those generated using the human and murine orthologs.

Methods

Materials

Murine IL-5 was purchased from R&D Systems (MN). Dibutyl phthalate, phthalic acid dinonyl ester, and polyvinylpyrrolidone were purchased from Sigma (MO). Excell 400 media and SF900II media were purchased from JRH Biosciences (KS) and GIBCO-BRL (MD), respectively. TF-1 cells were purchased from ATCC (MD). B13 cells were kindly provided by Dr Dilniya Fattah (GlaxoWellcome R&D). [¹²⁵I]hIL-5, [¹²⁵I]mIL-5 and [methyl-³H]thymidine were purchased from NEN (MA).

Cell culture

TF-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 U/ml penicillin and 10 μ g/ml streptomycin. A subclone (IL-5/TF-1) displaying a greater proliferative response to human IL-5 was selected following continuous culture in the presence of human IL-5. Optimal growth conditions were maintained by splitting the cultures every 3-4 days with medium containing 5 ng/ml IL-5. B13 cells were maintained in RPMI-1640 with 10% FBS containing 5 ng/ml murine IL-5.

Cloning guinea pig IL-5 cDNA

Guinea pigs were sensitized by i.p. injection of 5 mg ovalbumin in saline on day 1 and then 10 mg ovalbumin on day 3. On day 14 the animals were challenged with aerosolized ovalbumin (1 mg/ml in saline; 60-90 s) and 24 h later BAL was performed. mRNA was prepared from cells present in the BAL fluid. Adapter-ligated double stranded cDNA was synthesized from mRNA using a Marathon cDNA Amplification kit (Clontech Laboratories, Inc, CA).

Guinea pig IL-5 was cloned using nested PCR with the following primers:

Primer #1 was:

5'-CTCCGTCCAAAGGTAAAGCTGCACC-3'.

Primer #2 was:

5'-CCGGAATTCGGATCCTTGTAGCCATGAGGG-TGCTTCTGC-3'.

Primer #3 was:

5'-CGCGAAGCTTCCCAAATCTCTGGATACAAT-CAGCC-3'.

Primer #4 was:

5'-CCACATGGTACCCCATTAACATGTAC-3'.

Primer #2 incorporates EcoRI and BamHI sites and Primer #3 incorporates a HindIII site for subcloning into the pFASTBac1 vector sites. First round PCR amplification was done using 5 μ l of a 1:100 dilution of the product of a 1 μ g Marathon cDNA reaction as template. Primers #1 and #4 and Clontech's Advantage Polymerase mix were used in the first round PCR amplification which consisted of 30 cycles of 1.5 min at 94°C, 2 min at 55°C and 2 min at 68°C. One microliter of the 1st round PCR product was used as template in a second round reaction which used Primers #2 and #3. Conditions were the same as those described for first round PCR. The product of the second round PCR reaction was purified from an agarose gel using Qiagen's Qiaquick gel purification system and ligated into Novagen's pT7Blue T-Vector. A clone matching the published sequence (Genbank Accession #U34588) was identified and used to prepare expression vectors.

Expressing recombinant proteins using baculovirus expression system

The gpIL-5 cDNA was subcloned into the pFastBac-1 vector (GIBCO-BRL) downstream of the polyhedrin promoter. Recombinant baculovirus was obtained according to the Bac-to-Bac™ Baculovirus Expression System (Life Technologies, MD) protocols. High titer viral stocks were prepared in Sf9 cells using SF900II media. Optimal protein production was achieved using T.ni cells (3×10^6 cells/ml) in Excell 400 media with high titer virus at a multiplicity of infection of 5-10. Supernatants were harvested 72 h after infection and stored at 4°C.

Recombinant gpIL-5 $\alpha\beta_1$ and hIL-5 $\alpha\beta$ complexes were produced in Sf9 cells as previously described.¹⁸ Recombinant hIL-5 was expressed in Sf9 cells using procedures described by Brown *et al.*¹⁹

IL-5 purification

hIL-5 was purified by a combination of Q-Sepharose, hydroxylapatite and phenyl Sepharose column chromatography as described by Johanson *et al.*²⁰ gpIL-5 was purified in a single step by negative selection ion exchange chromatography on Q-Sepharose ff¹⁹ equilibrated with 10 mM Tris-HCl, pH 8. The gpIL-5 eluted in the exclusion volume while the remaining proteins were bound to the column. Contaminating lipids were precipitated by a 1:1 addition of 2 M $(\text{NH}_4)_2\text{SO}_4$ and then separated by centrifugation. The gpIL-5 preparation was then dialyzed against 50 mM NaH_2PO_4 , 150 mM NaCl, pH 7.0 to remove the $(\text{NH}_4)_2\text{SO}_4$.

Binding assay

Insect cells expressing either hIL-5 $\alpha\beta$ or gpIL-5 $\alpha\beta_1$ were processed for binding assays as previously described.¹⁸ Plasma membrane preparations (5 μg aliquots) were incubated with 50 pM [¹²⁵I]hIL-5 in assay buffer consisting of 25 mM Tris, pH 7.4 and 150 mM NaCl. Some samples also received various concentrations of unlabeled human, guinea pig, or murine IL-5. The samples were incubated at 23°C for 1 h and then vacuum filtered through 96-well GF/C filter plates (Packard, Meriden, CT) presoaked in 0.6% polyvinylpyrrolidone, 0.1% Tween-20. The filters were washed with 6 \times 1 ml aliquots of wash buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 0.02% BSA) and dried. Scintillation fluid was added and the amount of bound ligand measured using the TopCount Scintillation Counter (Packard). Specific binding was determined by subtracting the amount of binding observed in the presence of a 100-fold excess of unlabeled hIL-5. The membrane preparations containing hIL-5 $\alpha\beta$ and gpIL-5 $\alpha\beta_1$ showed greater than 90% specific binding when incubated with 50 pM [¹²⁵I]hIL-5. Equilibrium binding constants (K_d and K_i) and receptor densities (B_{max}) were determined using GraphPad (CA) InPlot software.

B13 cells were used to measure binding of various IL-5 orthologs to the mIL-5r. 1×10^6 cells were incubated with 50 pM [¹²⁵I]mIL-5 in RPMI-1640 media, 10% FBS, and 0.02% sodium azide. Some samples also received various concentrations of unlabeled murine, human or guinea pig IL-5. The samples were incubated at 23°C for 1 h and then overlaid onto 0.8 ml of a phthalate oil mixture (66% dibutyl phthalate, 34% phthalic acid dinonyl ester) in 1.5 ml microcentrifuge tubes. The tubes were centrifuged at $13,000 \times g$ for 3 min, snap frozen in a dry ice/methanol bath and cut just above the cell pellet. Cell-associated radioactivity was determined using a gamma counter (LKB Pharmacia, NJ).

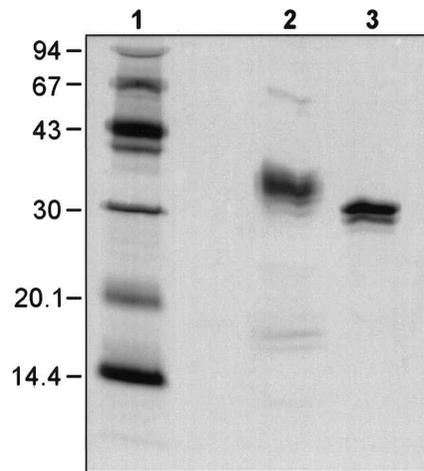


FIG. 1. SDS-PAGE analysis of gpIL-5 and hIL-5. The recombinant proteins were purified as described in Methods. Aliquots of gpIL-5 (0.05 μg ; lane 2) or hIL-5 (0.1 μg ; lane 3) were treated with SDS sample buffer in the absence of reducing agent and then run on a 20% SDS PhastGel (PhastSystem; Amersham Pharmacia Biotech). The gel was silver stained. The bands in lane 1 represent protein standards, with corresponding Mr noted in the margin.

Proliferation assay

TF-1 and B13 cells were washed in PBS then resuspended in growth media in the absence of serum. After 4 h of serum starvation the cells were seeded at 1.25×10^4 cells/well in a 96-well microtitre plate with various concentrations of cytokine or media alone. Proliferation was measured by pulsing cultures during the last 6 h of a 72 h culture period with 0.5 μCi of [methyl-³H]-thymidine per well. Samples were harvested on a 96-well UniFilter TM-96 GF/C filter plate (Packard, Meriden, CT). MicroScint-20 scintillation fluid (Packard) was added and incorporated [³H]-thymidine measured using the TopCount Microplate Scintillation Counter system (Packard).

Results

Cloning and expression of guinea pig IL-5

Mansour *et al.*¹¹ had previously reported the cloning of gpIL-5 from cells isolated from guinea pig spleen. We utilized a similar strategy, albeit using cells isolated by bronchoalveolar lavage (BAL) of ovalbumin-challenged animals. This method is known to yield an enriched preparation of eosinophils and Th2 cells, both of which produce IL-5.²¹⁻²³ A cDNA clone was obtained which had a predicted amino acid sequence identical to that published by Mansour *et al.*¹¹ A recombinant baculovirus construct was generated containing this clone and was used to express recombinant protein in *T. ni* insect cells.

Recombinant gpIL-5 was purified from the *T. ni* cell spent media using essentially a single step ion exchange chromatography procedure described by

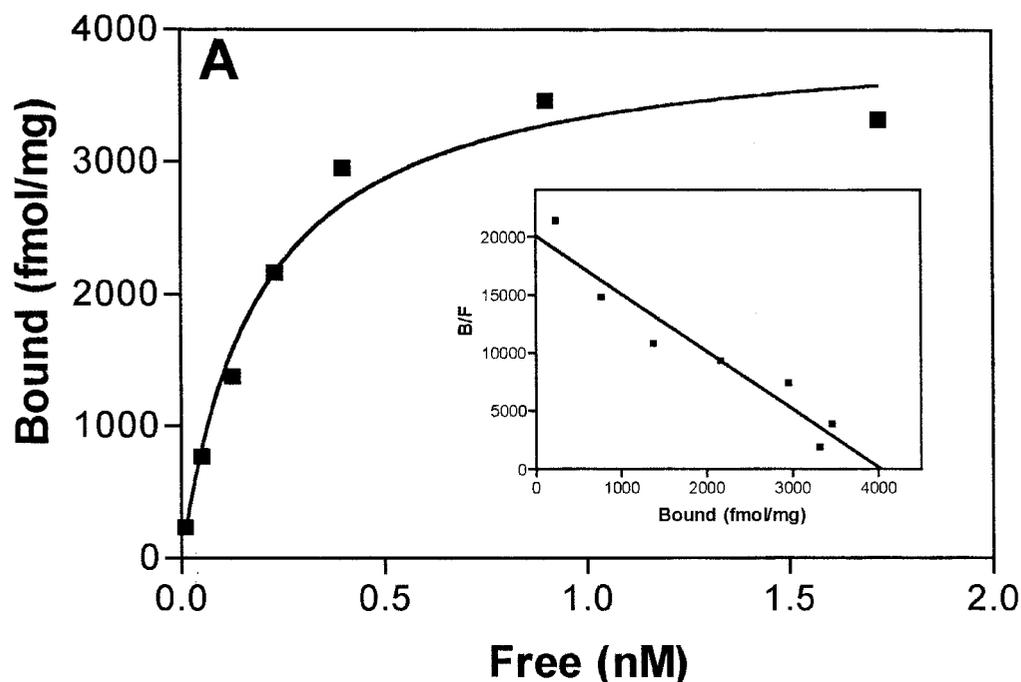


FIG. 2. Saturation analysis of [125 I]hIL-5 binding to Sf9 membranes expressing hIL-5 $\alpha\beta$. Plasma membranes from Sf9 cells infected with baculovirus containing hIL-5 $\alpha\beta$ were incubated with 30 pM–5 nM [125 I]hIL-5 under standard binding conditions. Data were analyzed as discussed in Methods and linearized according to the method of Scatchard (inset). Data represent the mean of triplicate determinations from a typical experiment.

Brown *et al.*¹⁹ As shown in Fig. 1, the purified protein migrates at the expected M_r on SDS-PAGE under non-reducing conditions, and is consistent with the known homodimeric form of the mature, biologically active protein. gpIL-5 migrates as a slightly higher M_r protein compared to human IL-5, presumably due to an additional glycosylation site (N59) plus a slightly larger molecular mass of the peptide sequence (13.36 *vs* 13.15 kDa). In the presence of reducing agent, both IL-5 preparations run at 15–18 kDa, which is consistent with the mature protein being comprised of disulfide-linked monomers (data not shown). The set of 2–3 bands seen with both preparations have been observed by others expressing guinea pig and human IL-5 in insect cells and represents differentially glycosylated forms of IL-5.^{11,19,24}

Binding to human and guinea pig IL-5r

The IL-5r is a heteromeric complex comprising an α and β subunit. The human IL-5r α and β subunits were co-expressed in Sf9 cells, and plasma membrane preparations derived from these cells were used to reconstitute high affinity binding with [125 I]hIL-5 (Fig. 2).¹⁸ Analysis of equilibrium binding data indicates a single population of saturable binding sites with a $K_d=220$ pM. This affinity is consistent with that shown for the binding of hIL-5 to human eosinophils.^{25–27} A similar high affinity binding site is observed with [125 I]hIL-5 and membrane preparations expressing

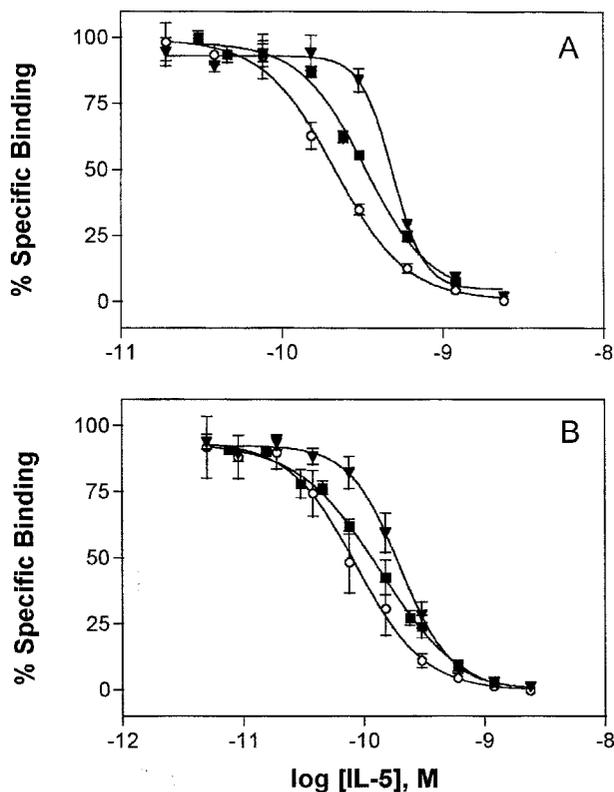


FIG. 3. Inhibition of [125 I]hIL-5 binding to human or guinea pig IL-5r by IL-5 orthologs. Membranes containing hIL-5 $\alpha\beta$ (A) or gpIL-5 $\alpha\beta$ (B) were incubated with 50 pM [125 I]hIL-5 in the presence of various concentrations of gpIL-5 (\blacktriangledown), hIL-5 (\blacksquare), or mIL-5 (\circ). Data are expressed as percent of control binding and represent the mean \pm SEM from three experiments performed in triplicate.

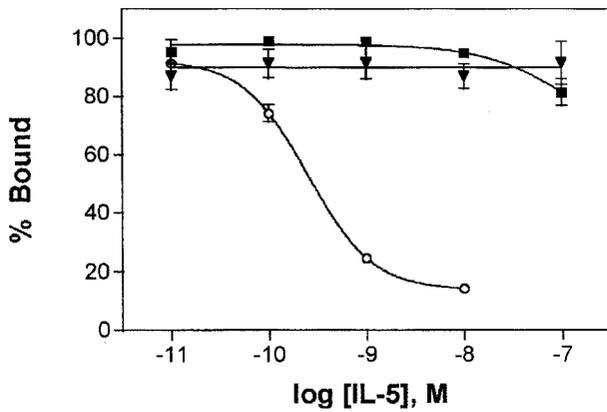


FIG. 4. Inhibition of [¹²⁵I]mIL-5 binding to B13 cells by IL-5 orthologs. Cells were incubated with 50 pM [¹²⁵I]mIL-5 in the presence of various concentrations of gpIL-5 (▼), hIL-5 (■), or mIL-5 (○). After 60 min, cells were centrifuged through an oil layer to separate bound from free ligand and the cell pellet processed as described in Methods. Data are expressed as percent of control binding and represent the mean±SEM from three experiments performed in triplicate.

guinea pig IL-5r α and β_1 subunits ($K_d = 110$ pM).¹⁸ These two preparations were used in displacement binding assays to determine whether gpIL-5 binds to gpIL-5r $\alpha\beta_1$ with an affinity similar to that seen with the human and murine ligand/receptor pairs and also to determine the relative affinities of guinea pig, murine and human IL-5 for the guinea pig and human IL-5 receptors.

As shown in Fig. 3a, the binding of [¹²⁵I]hIL-5 to hIL-5r $\alpha\beta$ was blocked with an excess of unlabeled hIL-5. The affinity of hIL-5 for hIL-5r $\alpha\beta$ determined by the displacement assay ($K_i = 470$ pM) agrees with that obtained in the saturation binding experiment described in Fig. 2 ($K_d = 220$ pM). mIL-5 displaced the binding of [¹²⁵I]hIL-5 to the hIL-5r $\alpha\beta$ preparation, and did so with a potency ($K_i = 170$ pM) similar to that seen with hIL-5. This is consistent with published results using human eosinophils as the source of IL-5r, indicating that human and murine IL-5 bind to the hIL-5r with similar affinity.²⁷ Finally, gpIL-5 also bound to the hIL-5r $\alpha\beta$ with high affinity ($K_i = 750$ pM), a value that was only 2 and 4.4-fold less potent than hIL-5 and mIL-5, respectively.

A similar set of competitive inhibition experiments was performed with plasma membranes from T.ni cells expressing gpIL-5r $\alpha\beta_1$ (Fig. 3b). gpIL-5 bound to its cognate receptor with high affinity, as demonstrated by blocking the binding of [¹²⁵I]hIL-5 with a $K_i = 160$ pM. This affinity is similar to that seen with the human IL-5 ligand/receptor pair. Both murine and human IL-5 also bound to this gpIL-5r preparation with high affinity, showing $K_i = 57$ and 97 pM, respectively. Thus, all three IL-5 orthologs bind to the recombinant guinea pig and human IL-5 receptors with high affinities, and differ from each other in potencies against either receptor preparation by less

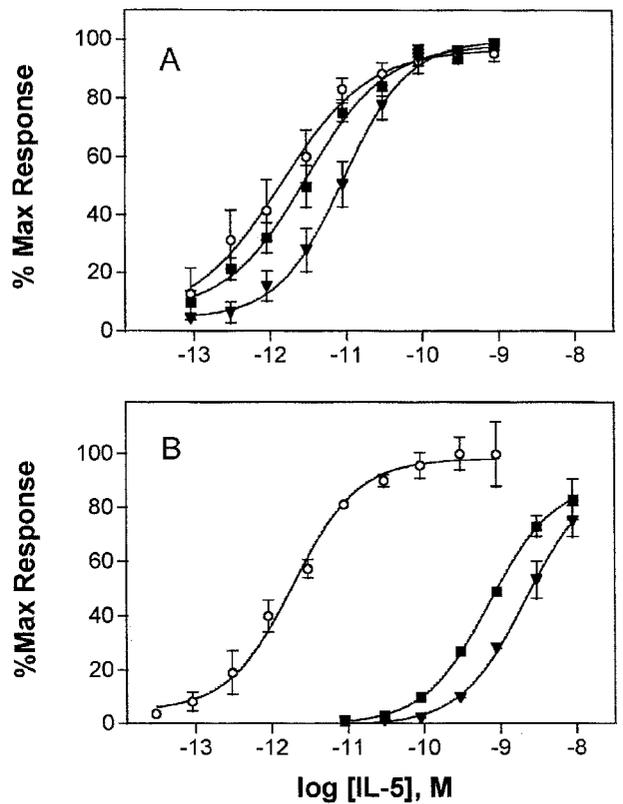


FIG. 5. Biological activity of IL-5 orthologs on human IL-5/TF-1 cells and murine B13 cells. Both IL-5/TF-1 (A) and B13 (B) cells were incubated with increasing concentrations of gpIL-5 (▼), hIL-5 (■), or mIL-5 (○) for 72 h and the proliferative response measured by incorporation of [³H]thymidine as described in Methods. Data represent the mean±SEM from three experiments performed in triplicate.

than 5-fold. For both preparations of receptors, these ligands demonstrated the same rank order potency with mIL-5 > hIL-5 > gpIL-5.

Binding to murine IL-5r

B13 cells are a murine Ly1+ pre-B cell line previously shown to express receptors for mIL-5 and to proliferate in response to this cytokine.^{28,29} These cells were used to determine the relative affinities of the IL-5 orthologs for the murine receptor. As shown in Fig. 4, binding of [¹²⁵I]mIL-5 can be displaced with unlabelled mIL-5 but not hIL-5. Only at the highest concentration of hIL-5 tested was there noticeable displacement of [¹²⁵I]mIL-5. This is consistent with previous reports indicating that mIL-5 binds to mIL-5r with a 400–5000-fold higher affinity than hIL-5.^{30,31} Interestingly, the results with gpIL-5 closely resembled that of hIL-5 rather than mIL-5, i.e. gpIL-5 was unable to displace [¹²⁵I]mIL-5 from the mIL-5r. These results indicate that high affinity binding seen with mIL-5 is not shared with all other rodent IL-5 orthologs.

Biological activity of recombinant gpIL-5

The biological activity of gpIL-5 was assessed using a subclone of human TF-1 cells, (IL-5/TF-1), previously isolated by long-term culture in hIL-5 and showing a stronger proliferative response to hIL-5 compared to the parental line. Similar subclones have been isolated by others using the same strategy.³² As shown in Fig. 5a, these cells proliferate in response to all three IL-5 orthologs, with gpIL-5 showing slightly less activity compared to murine and human IL-5 (EC_{50} = 9.1 vs 1.4 and 2.9 pM, respectively). The IL-5 orthologs induce proliferation at concentrations below their K_d for the hIL-5r which indicates the presence of spare receptors on these cells. All three IL-5 orthologs produced the same maximal proliferative response with the IL-5/TF-1 cells and did so with the same rank order potency observed in the binding experiments.

In contrast to the results with IL-5/TF-1 cells, there was a substantial difference in the ability of guinea pig, human and murine IL-5 to induce proliferation of B13 cells (Fig. 5b). mIL-5 was 350–1000-fold more potent than human or guinea pig IL-5. This separation in potency of the biological responses by the murine receptor is consistent with the different binding affinities for this receptor. All three IL-5 preparations had similar intrinsic activities on the mIL-5r, i.e. were capable of eliciting a maximal response, but their relative potencies reflected the differences in affinity for this receptor. Thus, only mIL-5 can bind and activate the mIL-5r with high affinity.

Discussion

Delineating the biological interaction of guinea pig IL-5 with its receptor would prove useful for comparison to the human ligand/receptor pair, particularly for relating the molecular properties of hIL-5r antagonists to their *in vivo* profile in guinea pig models of allergic airway disease. Although gpIL-5 and gpIL-5r have been cloned and expressed in biologically active forms,¹¹ their interaction has not been evaluated. In this paper we describe the binding affinity of recombinant gpIL-5 to its receptor and compare its cross-species affinity and agonist activity with that of human and murine IL-5.

As shown in Fig. 3, guinea pig IL-5 bound with high affinity to recombinant gpIL-5 $\alpha\beta_1$ expressed in Sf9 cells. It bound with a similar high affinity to hIL-5 $\alpha\beta$, but did not demonstrate appreciable binding to mIL-5r on B13 cells. Under the conditions used in the mouse B13 cell binding assay, gpIL-5 showed no inhibition of binding at a concentration 1000-fold higher than that required for displacement with mIL-5, indicating that gpIL-5 has at least 1000-fold lower affinity for the murine receptor compared to mIL-5. Human IL-5 showed a binding profile that was remarkably similar to gpIL-5 across the three receptor

preparations, namely high affinity binding to hIL-5r and gpIL-5r, but not mIL-5r. Furthermore, the affinity of gpIL-5 for its cognate receptor is similar to that observed with the human IL-5 ligand/receptor pair (Fig. 3).^{12,33}

Recombinant gpIL-5 activated the hIL-5r on human TF-1 cells, stimulating a maximal proliferative response with a potency similar to that seen with hIL-5. gpIL-5 was also a full agonist for the mIL-5r expressed on B13 cells, but was 1000-fold less potent than mIL-5. Thus, the biological potency of gpIL-5 correlated with its receptor-binding affinity. A similar correlation between receptor binding affinity and agonist potency was observed with hIL-5 and is consistent with previous reports.^{13,30}

Both murine/human chimeric polypeptides and alanine scanning mutagenesis have been used to identify the key charged residues on hIL-5 involved in binding to the hIL-5r α and β subunits.^{14,16,31,34,35} These studies have shown that amino acids E88, R90 and E109 are involved in interactions with the receptor α chain while E12 is critical for β chain binding and receptor activation. All of these amino acids are identical for the human, murine and guinea pig IL-5 orthologs. Site directed mutagenesis studies also identified the residues on hIL-5 responsible for the very weak binding affinity to mIL-5r.¹³ Replacing two amino acids on hIL-5 with the murine counterparts, K84E and N108S, produced a mutant protein with a biological activity similar to mIL-5. Single mutations had biological activities in between mIL-5 and hIL-5. This indicates that both K84 and N108 are key residues on hIL-5 which prevent high affinity binding to mIL-5r. Interestingly, gpIL-5 does not bind to mIL-5r with high affinity, and like hIL-5 contains asparagine at residue 108. However, gpIL-5 contains glutamine at position 84, not lysine like hIL-5. Therefore, high affinity binding to mIL-5r likely requires an acidic side chain (E) at residue 84 for neither a basic side chain (K) nor an amide side chain (Q) are tolerated. The amino acid sequence of both rat and gerbil IL-5 are known, and both contain E84 and S108 like the murine ortholog. Thus, gpIL-5 appears to be an outlier within the rodent family, having an amino acid sequence at critical domains that are more similar to hIL-5 and demonstrating cross-species biological activity that mimics the human ortholog.

The data in this report indicates that guinea pig and human IL-5 are similar in their cross-species profiles: both are relatively poor agonists on the mIL-5r yet bind human and guinea pig IL-5r with high affinity. Murine IL-5 binds with high affinity to all three IL-5 receptors. These data support the use of guinea pig models to evaluate the *in vivo* profile of compounds designed to block the interaction of hIL-5 with its cognate receptor. The identification and optimization of such compounds remains a substantial challenge.

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