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Rational design of a receptor super-antagonist of human interleukin-6

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Interleukin-6 (IL-6) is a differentiation and growth factor for a variety of cell types and its excessive production plays a major role in the pathogenesis of multiple myeloma and post-menopausal osteoporosis. IL-6, a four-helix bundle cytokine, is believed to interact sequentially with two transmembrane receptors, the low-affinity IL-6 receptor (IL-6Rα) and the signal transducer gp130, via distinct binding sites. In this paper we show that combined mutations in the predicted A and C helices, previously suggested to establish contacts with gp130, give rise to variants with no bioactivity but unimpaired binding to IL-6Rα. These mutants behave as full and selective IL-6 receptor antagonists on a variety of human cell lines. Furthermore, a bifacial mutant was generated (called IL-6 super-antagonist) in which the antagonist mutations were combined with amino acid substitutions in the predicted D helix that increase binding for IL-6Rα. The IL-6 super-antagonist has no bioactivity, but improved first receptor occupancy and, therefore, fully inhibits the wild-type cytokine at low dosage. The demonstration of functionally independent receptor binding sites on IL-6 suggests that it could be possible to design super-antagonists of other helical cytokines which drive the assembly of structurally related multisubunit receptor complexes.

Key words: interleukin-6 / multiple myeloma / receptor antagonist

Introduction

Interleukin-6 (IL-6) is a factor controlling growth and/or differentiation of cells of the immune system, osteoclasts and hepatocytes (Van Snick, 1990). In contrast to its physiological role, IL-6 overproduction has been suggested to participate in the pathogenesis of several diseases. These include multiple myeloma, post-menopausal osteoporosis and chronic autoimmune diseases, where a constant association has been shown between disease state and increased production of the cytokine (Bataille *et al.*, 1989; Hirano *et al.*, 1990; Poli *et al.*, 1994). Moreover, IL-6 neutralizing antibodies have been shown to exert a short-term beneficial effect both in animal models of these pathologies and in human pre-clinical trials (Vink *et al.*, 1990; Klein *et al.*, 1991; Jilka *et al.*, 1992; Kiberd, 1993; Wendling *et al.*, 1993). For these reasons it is generally believed that potent IL-6 receptor antagonists will be valuable therapeutic tools.

IL-6 belongs to a family of cytokines, including leukaemia inhibitory factor (LIF), oncostatin M (OM), ciliary neurotrophic factor (CNTF) and interleukin 11 (IL-11), which all assemble receptor complexes formed by a cytokine-specific receptor subunit and the gp130 transmembrane protein as an essential signalling component (Kishimoto *et al.*, 1994). In particular, IL-6 assembles a multiprotein receptor complex via sequential interaction with the low-affinity IL-6 receptor α (IL-6Rα) and the signalling subunit gp130 (Taga *et al.*, 1989; Hibi *et al.*, 1990). On these grounds, IL-6 variants which are still able to associate with IL-6Rα but have lost the capacity to recruit gp130 are predicted to be efficient and specific receptor antagonists. A rational design of these antagonists could start from three-dimensional data of the hormone complexed with its receptors. However, since this information does not exist for the components of the IL-6–receptor complex, we used molecular modelling for the basic design of our receptor antagonist.

IL-6 has been proposed to fold, like growth hormone (GH), as a bundle of four α-helices (A, B, C and D) (Bazan, 1991). Furthermore, both IL-6Rα and gp130 contain a cytokine binding domain whose global fold is believed to be similar to that present in the GH receptor (Bazan, 1990). In GH, two distinct and separate surface areas have been identified which promote sequential interaction with the components of its homodimeric receptor (Cunningham *et al.*, 1991). Superposition of a three-dimensional model of human IL-6 (hIL-6) onto the $GH(GHbp)_2$ X-ray structure (De Vos *et al.*, 1992) led us to speculate that in the case of IL-6, interactions between the cytokine and its receptors also involve two surfaces located on opposite sides of the IL-6 molecule: Site 1 (formed by the C-terminal part of helix D and in part by the AB loop) interacting with IL-6Rα and Site 2 (formed by a limited number of exposed residues on helix A and helix C) where the signal transducer gp130 binds (Savino *et al.*, 1994). This model is strongly supported by site-directed mutagenesis studies of human IL-6 performed by several groups, showing that mutations of the IL-6 C-terminus (part of Site 1) strongly affect interaction with the IL-6Rα chain, but leave the conformation of the cytokine sustantially unaltered (Brakenhoff *et al.*, 1990; Leebeek *et al.*, 1992; Fontaine *et al.*, 1993; Savino *et al.*, 1993). Moreover, mutation of residues 31 and 35 in helix A (mutant Y31D/G35F), predicted by the model as involved in the interaction with gp130 (Site 2), produced variants that bind normally to IL-6Rα, whose *in vitro* interaction with gp130 is severely impaired and which

display low levels of residual activity only at very high concentrations (Savino *et al.*, 1994). This study demonstrated that it is possible to dissociate IL-6Rα binding from gp130 recruitment and signal transduction.

The residual biological activity shown by the helix A mutant Y31D/G35F and its behaviour as a partial antagonist suggest, however, that some residual interaction with gp130 still takes place, which is below the threshold of detection of our *in vitro* binding assay (Savino *et al.*, 1994). We therefore decided to identify additional residues of Site 2. In this paper we report that the combined mutagenesis of helix C and helix A residues which include Y31D/G35F totally abolishes any residual biological activity and gives rise to full and highly specific IL-6 receptor antagonists on a large variety of human cell lines. Furthermore, in order to assess that Site 1 and Site 2 function independently in receptor assembly, we combined the antagonist mutations of Site 2 with mutations of Site 1 which increase the affinity for IL-6Rα (Savino *et al.*, 1993). As predicted by the model, this bifacial mutant behaves as a super-antagonist, as it inhibits IL-6 bioactivity at low dosages. The therapeutic potential of the IL-6 super-antagonist for the treatment of IL-6-dependent diseases will also be discussed.

Results

Molecular predictions

In a recent study we demonstrated that a two amino acid substitution of exposed residues in helix A (Y31D/G35F) does not decrease the binding for IL-6Rα, but the resulting IL-6−IL-6Rα complex is unable to interact *in vitro* with a soluble recombinant form of gp130 produced in CHO cells. However, since the same mutant still maintains a residual biological activity on hepatoma cells and behaves only as a partial IL-6 receptor antagonist, we assumed that it would be able to trigger a marginal recruitment of gp130 on cells which is below the limit of detection of our *in vitro* binding assay (Savino *et al.*, 1994). Therefore, while it was clear that Y31 and G35 are part of Site 2, additional residues must also compose this site and only identifying them through site-directed mutagenesis would allow us to arrive at total inhibition of of gp130 recruitment and loss of bioactivity.

A closer examination of the three-dimensional model of IL-6 in comparison with the published structure of growth hormone (GH) and a computation of all available mutagenic studies on these two molecules suggests that, besides broad similarities between the two systems, the relative distribution of the residues making contact with the second receptor is slightly different in GH and in IL-6 (Figure 1). In fact, residues of GH demonstrated to be important for interaction with the second receptor, both by X-ray crystallography (De Vos *et al.*, 1992) and site-directed mutagenesis (Cunningham *et al.*, 1991; Fuh *et al.*, 1992), cluster in a region of IL-6 populated by residues not relevant for interaction with gp130 (Brakenhoff *et al.*, 1989; Savino *et al.*, 1994) (Figure 1). Furthermore, the topological localization of Y31 and G35 in IL-6 suggests that the area making important contact with the second receptor is shifted, relative to GH, one to two helical turns towards the end of helix A and the beginning of helix C respectively. It is reasonable to hypothesize, therefore,

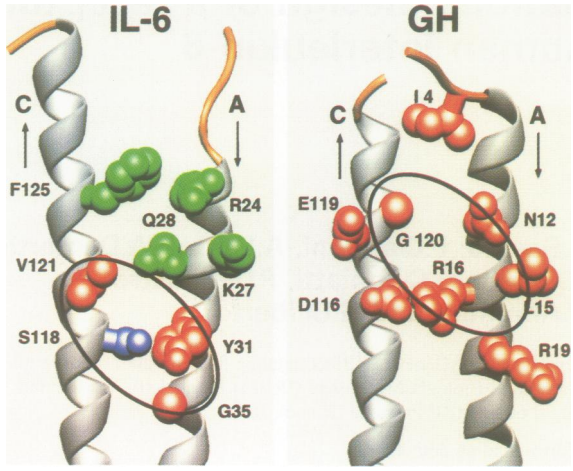

Fig. 1. Schematic representation of IL-6 and GH helices A and C. Residues previously shown to be important for Site 2 receptor recognition in GH by X-ray structure (De Vos *et al.*, 1992) and in IL-6 by mutagenesis (Savino *et al.*, 1994) are coloured red for both IL-6 and GH. Green indicates IL-6 residues that, by strict analogy with GH, should contribute to Site 2, but which can instead be mutated without affecting biological activity (Brakenhoff *et al.*, 1989; Savino *et al.*, 1994). Considering the binding site of hGHbp W104 and W169 as the centre of GH Site 2 (black circle), the identification of IL-6 Y31, G35 and V121 as part of the gp130 recognition surface suggests a shift of IL-6 Site 2 (black circle) relative to GH towards the end of helix A and the beginning of helix C respectively. As a consequence, Ser118 (indicated in blue) is now predicted to be in the centre of IL-6 Site 2 and was therefore mutagenized. Graphics were produced using the program RIBBONS (Carson, 1987).

that S118, the residue on helix C predicted by the model to be in spatial proximity with Y31 and G35 (Figure 1) might be another crucial component of Site 2.

IL-6 mutagenesis

Serine 118, a small, polar amino acid, was mutated to arginine, leucine or phenylalanine, to change both its size and characteristics. The resulting mutants were examined either individually or in combination with the mutation V121D, the only helix C mutation which slightly, but significantly, reduces biological activity (Savino *et al.*, 1994). Mutants were tested for biological activity (transcriptional activation of a transfected IL-6 inducible promoter) in the human hepatoma Hep3B cell line and in an *in vitro* receptor binding assay (summarized in the upper part of Table I). Interestingly, mutant IL-6 S118R/V121D has characteristics very similar to the partial antagonist IL-6 Y31D/G35F, namely a strongly increased EC_{50} and inability to reach maximal stimulation while maintaining receptor binding capacity almost intact.

We then combined mutations in S118 and V121 on helix C with the helix A Y31D/G35F and generated, amongst others, mutants DFRD, DFFD and DFLD (Table I). Tests of their biological activity and receptor binding show that all three mutants are completely silent, even at the highest concentration tested (4 μg/ml), while maintaining their binding to IL-6Rα substantially unaltered (Table I). From these results we can conclude that residues Tyr31/Gly35 in helix A and Ser118/Val121 in helix C, which our model predicts as forming a continuous patch on the IL-6 surface (Figure 1), all participate in triggering

Table I. Biological activity and receptor binding of IL-6 mutants

Mutations				Biological activity		sIL-6Rα binding (% of wt)
Helix A		Helix C		EC_{50} (ng/ml)	Maximal activity (% of wt)	
Y31	G35	S118	V121	0.8 ± 0.2	100	100
D	F			40 ± 9	51 ± 7	84 ± 9
			D	1.4 ± 0.5	100 ± 5	78 ± 2
		R		1.2 ± 0.2	100 ± 9	81 ± 18
		L	D	2.2 ± 0.2	100 ± 4	92 ± 34
		R	D	22.8 ± 3.3	66 ± 6	66 ± 5
D	F	L		57 ± 9.7	58 ± 7	20 ± 2
D	F	R		14.7 ± 3.1	63 ± 7	66 ± 2
D	F	R	D[a]	>4000	0	97 ± 15
D	F	F	D[b]	>4000	0	58 ± 13
D	F	L	D[c]	>4000	0	69 ± 18

[a]This mutant is referred to in the text as DFRD.
[b]This mutant is referred to in the text as DFFD.
[c]This mutant is referred to in the text as DFLD.
Biological activity is measured as activation of the IL-6-inducible C-reactive protein (CRP) gene promoter transfected into human Hep 3B hepatoma cells (Gregory *et al.*, 1994). The EC_{50} is the concentration of each mutant which gives 50% of the wt IL-6 maximal stimulation and is determined by dose–response curves as previously described (Savino *et al.*, 1994). The receptor binding activity was determined as previously described (Savino *et al.*, 1994) The first row shows the wt IL-6 sequence in positions 31, 35, 118 and 121. For each mutant, where no change is indicated the wild-type residue is present.

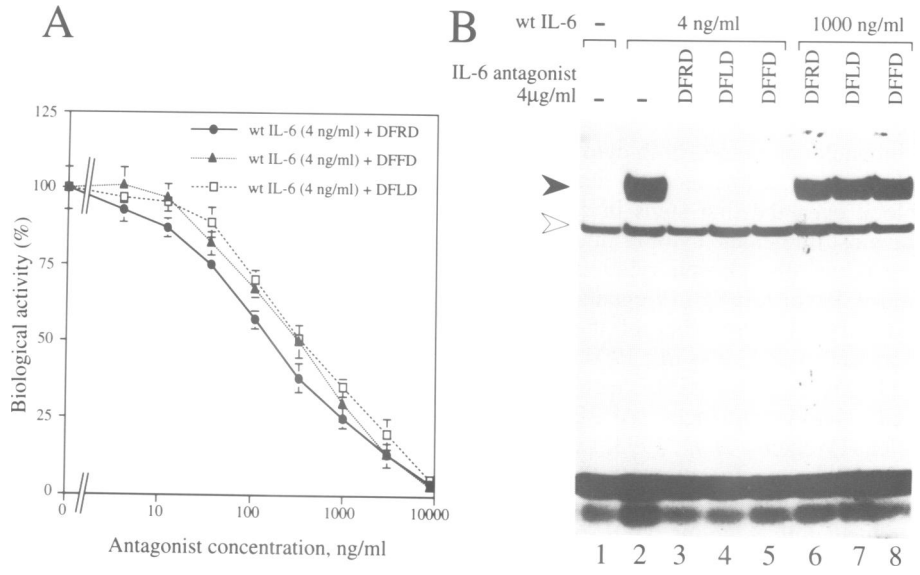

Fig. 2. IL-6 mutants inhibit wt IL-6 activity on human Hep3B hepatoma cells. (**A**) Human Hep3B cells were transfected with the IL-6-inducible CRP gene promoter fused to a secretable form of alkaline phosphatase (SEAP) as reporter gene (Gregory *et al.*, 1994) and induced with 4 ng/ml wt IL-6 in the presence of increasing concentrations of each IL-6 mutant, as indicated. SEAP activity was quantified (Gregory *et al.*, 1994) and expressed as a percentage of the transcriptional efficiency in cells incubated with 4 ng/ml wt IL-6 alone. (**B**) Hep3B cells were incubated (15 min) with no cytokine (lane 1), with wt IL-6 at 4 ng/ml (lanes 2–5) or at 1000 ng/ml (lanes 6–8) in the absence (lane 2) or presence (lanes 3–8) of the various IL-6 mutants (as indicated) at 4 μg/ml. Whole cell extracts were prepared as described in Materials and methods. APRF binding to the high-affinity SIE m67 oligonucleotide and electromobility shift assays were performed essentially as previously described (Sadowski and Gilman, 1993). All IL-6 mutants fully inhibit IL-6-induced APRF activation (filled arrowhead) and the inhibition is reversed by large amounts of wt IL-6 (lanes 6–8). The open arrowhead indicates the position of a non-specific signal.

signalling events in IL-6-stimulated cells, most probably through direct interaction with gp130.

Mutant antagonism of wild-type IL-6 action on Hep3B

The same mutants were tested for their capacity to antagonize wild-type (wt) hIL-6 on human hepatoma cells. Hep3B cells were incubated with 4 ng/ml wt hIL-6 and increasing amounts of each mutant (Figure 2A). Full inhibition of wt hIL-6 is reached at a 2000 molar excess; 50% inhibition is obtained at concentrations of DFRD lower than those needed for both DFFD and DFLD (44- versus 88- and 97-fold molar excess over wt IL-6 respectively). Interestingly, these values are in line with the mutant affinity for IL-6Rα (see Table I). Finally, inhibition is not the result of toxicity, because it could be reversed by adding large amounts (1 μg/ml) of wt hIL-6 (data not shown).

The biological assay which measures the activation of a transfected IL-6-dependent promoter is the result of a

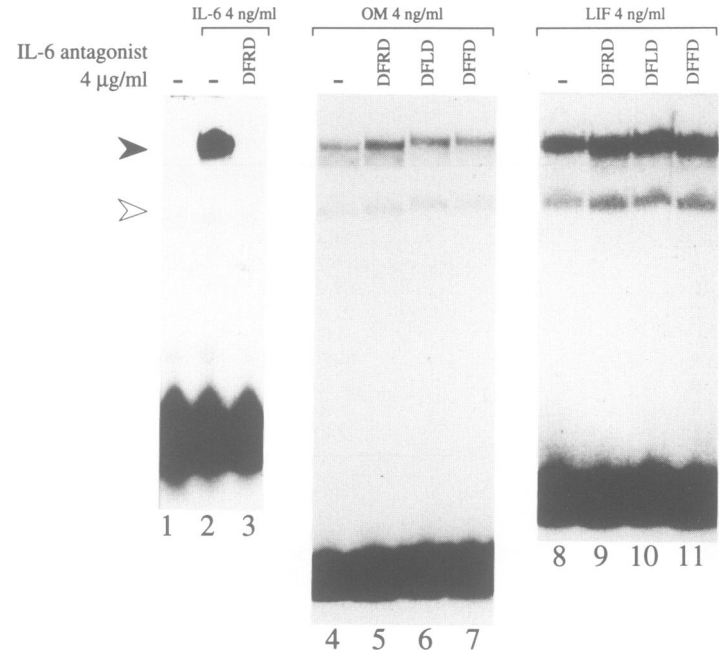

Fig. 3. The three receptor antagonists are specific for IL-6. HepG2 cells were incubated for 15 min with no cytokine (lane 1) or with 4 ng/ml IL-6 (lanes 2–3), OM (lanes 4–7) or LIF (lanes 8–11) in the absence or in the presence of the various IL-6 antagonists (as indicated) at 4 μg/ml. APRF activation was monitored by incubating 5 μg whole cell extracts with labelled α2M APRE, as previously described (Wegenka *et al.*, 1993). Complexes were resolved on 5% polyacrylamide−7.5% glycerol−0.25× TAE (1× TAE: 40 mM Tris, pH 7.8, 37 mM sodium acetate, 1.1 mM EDTA) gels. Open and filled arrowheads as in Figure 2.

cascade of multiple intracellular events, both cytoplasmic and nuclear. On the other hand, a true receptor antagonist should be able to block even the first steps in cytokine signalling. One of the most immediate cytoplasmic events after IL-6 stimulation is tyrosine phosphorylation of the acute phase transcription factor (APRF) (Wegenka *et al.*, 1993; Lütticken *et al.*, 1994), a member of the STAT family of transcription factors (Akira *et al.*, 1994; Zhong *et al.*, 1994). Upon phosphorylation, APRF acquires the ability to bind specific DNA sequences (APREs) and migrates into the nucleus (Wegenka *et al.*, 1993; Lütticken *et al.*, 1994). We tested whether the mutants described above would be able to inhibit APRF activation. Human Hep3B cells were treated with 4 ng/ml wt IL-6 in the presence or absence of the three mutants at 4 μg/ml. After 15 min, whole cell extracts were prepared and APRF activation was monitored by gel retardation. APRF activation is completely inhibited by the three antagonists (Figure 2B, lanes 2–5) and inhibition is reversed by a large excess of wt hIL-6 (Figure 2B, lanes 6–8).

The IL-6 antagonists are highly specific

The discovery that the members of the IL-6 cytokine family (IL-6, LIF, OM, CNTF and IL-11) all use the transmembrane protein gp130 as common signal transducer furnished a clear explanation for their functional redundancy on various cell types (Kishimoto *et al.*, 1994). In fact, both gp130 homodimerization driven by IL-6 (Murakami *et al.*, 1993) and gp130−LIFR β-subunit heterodimerization driven by LIF, OM and CNTF (Davis *et al.*, 1993) lead to activation of common signalling pathways, which include the phosphorylation of APRF (Stahl *et al.*, 1994).

We expected that one characteristic of the three IL-6 antagonists DFRD, DFFD and DFLD is that once bound to cell surface IL-6Rα, they would not be able to prevent gp130 from interacting with other cytokines of the same family: the net effect would be failure to antagonize OM and LIF. To test this idea we chose human hepatoma HepG2 cells, which are not only responsive to IL-6, but

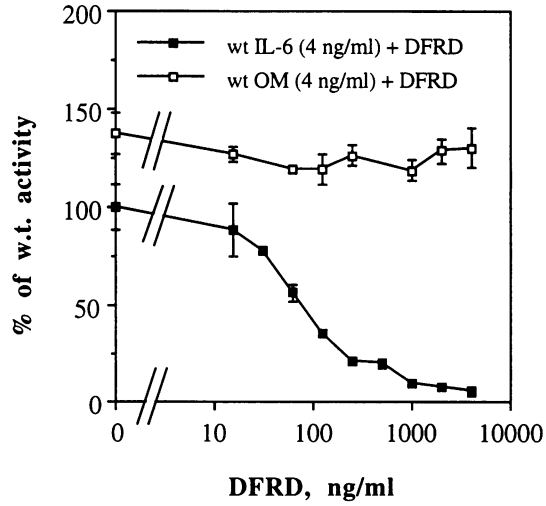

Fig. 4. The mutant DFRD inhibits IL-6-induced, but not OM-induced, CPR promoter transcriptional activation. Human HepG2 cells were transfected with the IL-6-inducible CRP gene promoter fused to the SEAP reporter gene (Gregory *et al.*, 1994) and induced with 4 ng/ml wt IL-6 (filled symbol) or OM (open symbol) in the presence of increasing concentrations of the IL-6 mutant DFRD, as indicated. SEAP activity was quantified by a colourimetric assay, as previously described (Gregory *et al.*, 1994), and expressed as a percentage of the transcriptional efficiency in cells incubated with 4 ng/ml wt IL-6 alone. The other two mutants (DFFD and DFLD) behave similarly to DFRF (data not shown).

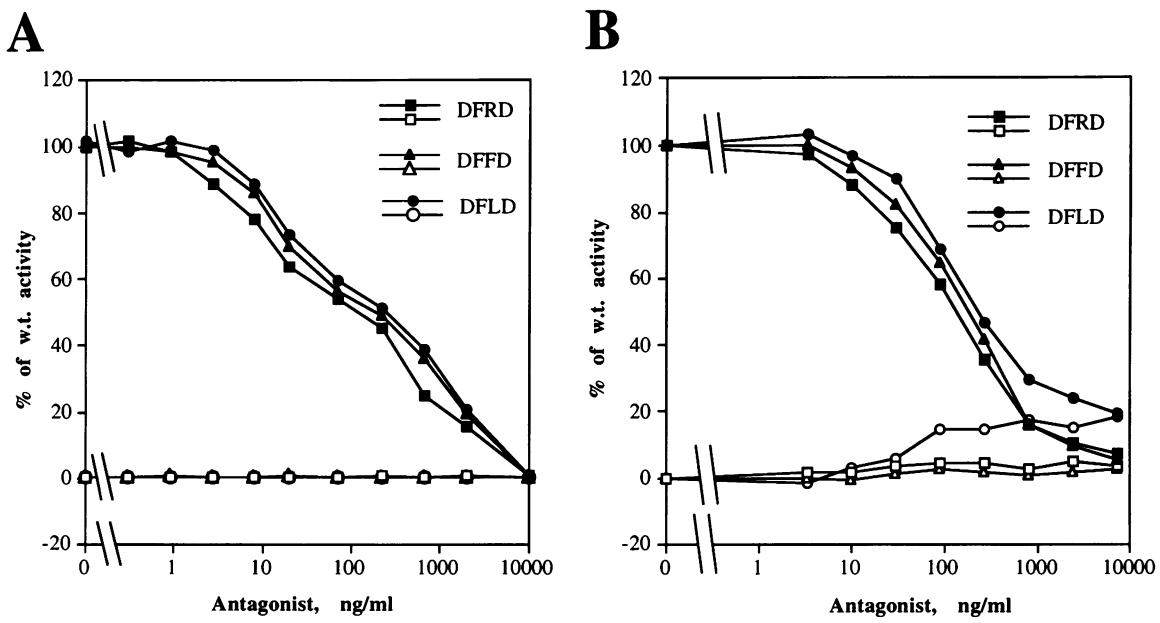

Fig. 5. The three IL-6 antagonists inhibit wt IL-6 on melanoma and myeloma cells. (**A**) A375 melanoma cells were incubated with increasing concentrations of DFRD (squares), DFLD (circles) and DFFD (triangles) in the absence (open symbols) or presence (filled symbols) of 4 ng/ml wt IL-6. The effect of wt IL-6 and of the IL-6 antagonists, both alone and in combination, on A375 growth was determined by crystal violet staining (Horn *et al.*, 1990). (**B**) XG-1 myeloma cells were cultured in 96-well microtititre plates with various concentrations of each IL-6 antagonist, in the presence (filled symbols) or absence (open symbols) of 0.1 ng/ml wt IL-6. Symbols are as in (A). After 7 days of culture, cell numbers were evaluated by colourimetric determination of hexosaminidase levels (Landegren, 1984) and expressed as a percentage of the number of cells incubated with 0.1 ng/ml IL-6 alone, after subtraction of the background reading given by control, non-induced cells. In both cases each point is the average of duplicate measurements. The standard error is not shown for clarity of illustration; it was on average between 1 and 5% of the absolute value of each point.

also to OM and LIF, because they express the LIF receptor β-subunit (Baumann *et al.*, 1993).

APRF induction by IL-6 is inhibited in HepG2 by DFRD, DFFD and DFLD at a 1000-fold molar excess (Figure 3 and data not shown). The transcription factor APRF is activated when HepG2 cells are stimulated with OM (Figure 3, lane 4) or LIF (Figure 3, lane 8), but in both cases the three mutants completely fail to antagonize APRF activation (Figure 3, lanes 5–7 and 9–11). Likewise, OM-induced transcriptional activation of the CRP promoter transfected into HepG2 cells is not inhibited by the most potent antagonist, DFRD (Figure 4). It can be concluded, therefore, that these antagonists are highly specific for IL-6.

The mutants inhibit IL-6 activity on a variety of IL-6-responsive human cell lines

The mutants' antagonistic action is also effective on the non-hepatoma human cell lines XG-1and A375, the latter being a melanoma cell line where IL-6 causes growth inhibition (Bruce *et al.*, 1992). When tested on A375, DFRD, DFFD and DFLD have no detectable bioactivity and fully antagonize IL-6, therefore inhibiting the anti-proliferative effect of the cytokine (Figure 5A).

XG-1 cells derive from a multiple myeloma, are strictly IL-6-growth dependent and are an excellent model for *in vitro* testing of the effect of IL-6 antagonists on the growth of multiple myeloma (Jourdan *et al.*, 1991). These cells are very sensitive to IL-6, with an EC_{50} of 40 pg/ml (Jourdan *et al.*, 1991; data not shown), a value ~20-fold lower than that observed in Hep3B cells (Table I). On XG-1, DFRD and DFFD are completely inactive, whereas DFLD retains 10–12% residual activity (Figure 5B). Furthermore, all three mutants antagonize hIL-6 in a dose-dependent manner, even though DFLD is unable to inhibit >90% (Figure 5B). However, due to the extreme sensitivity of XG-1 cells, higher amounts are required for biological antagonism (50% growth inhibition was reached at 1860-, 2100- and 3100-fold molar excess for DFRD, DFFD and DFLD respectively). This suggests that, despite the full antagonism shown by DFRD and DFFD, these molecules would be of little or practically no value in the treatment of IL-6-dependent pathologies like multiple myeloma.

Generation of an IL-6 super-antagonist

The present model of IL-6–receptor interaction (Savino *et al.*, 1994) suggests that Sites 1 and 2 function independently in receptor interaction. This idea is strengthened by the observation that of the three receptor antagonists, DFRD, which has the highest affinity for IL-6Rα, is the most potent (Figures 2 and 5). A prediction of the model is that further increasing the affinity of the antagonist for IL-6Rα would increase its potency without any increment in bio-activity, an effect that we call super-antagonism.

In a previous saturation mutagenesis study of IL-6 Site 1 we were able to generate an IL-6 variant, with a Ser→Arg substitution at position 176, showing a 3-fold increased affinity for IL-6Rα (Savino *et al.*, 1993). We also recently identified additional substitutions in the same region, Q175I and Q183A, that when present with S176R in the triple substitution mutant IL-6/IRA cause a 4.5-fold affinity increase (A.Cabibbo and G.Ciliberto, in preparation). These three substitutions in the D helix were inserted in mutant DFRD generating mutant IL-6 SAnt (for super-antagonist).

Table II. Antagonistic potency of IL-6 DFRD and IL-6 SAnt

Mutant	sIL-6Rα binding (% of wt)	Receptor antagonist molar excess needed to reach 50% inhibition				
		Hep3B[a]	HepG2[a]	A375[a]	CESS[b]	XG-1[c]
DFRD	97 ± 15	44 ± 9	34.5 ± 9	25 ± 4	80 ± 10	1860 ± 295
SAnt	406 ± 60	5 ± 0.4	3.2 ± 0.3	3 ± 0.5	10 ± 3	227 ± 25

[a]Hep3B, HepG2 and A375 cells were stimulated with 4 ng/ml wt IL-6.
[b]CESS cells were stimulated with 2 ng/ml wt IL-6.
[c]XG-1 cells were stimulated with 0.1 ng/ml wt IL-6.
The molar excess of IL-6 DFRD and of IL-6 SAnt necessary to give 50% inhibition in various bioassays was calculated from inhibition curves like the ones shown in Figures 2A and 4–6.

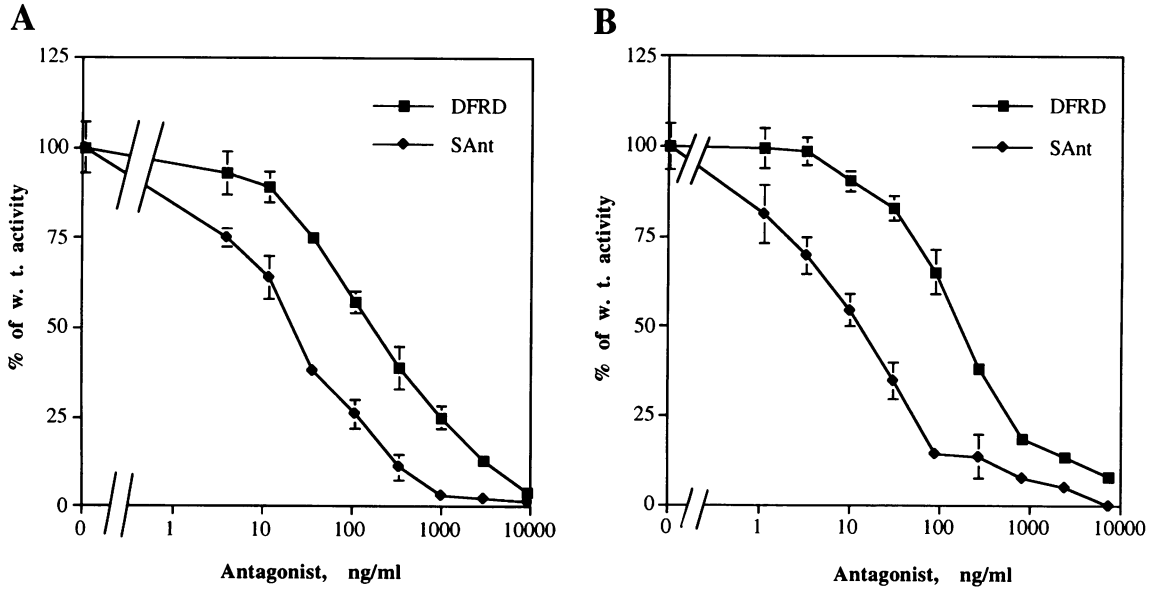

Fig. 6. IL-6 SAnt is a more potent antagonist than DFRD. (**A**) Human Hep3B cells were transfected with the IL-6-inducible CRP–SEAP construct (Gregory *et al.*, 1994) and induced with 4 ng/ml wt IL-6 in the presence of increasing concentrations of IL-6 DFRD and IL-6 SAnt, as indicated. SEAP activity was quantified (Gregory *et al.*, 1994) and expressed as a percentage of the transcriptional efficiency in cells incubated with 4 ng/ml wt IL-6 alone. (**B**) XG-1 cells were cultured and induced as in Figure 5B. After 7 days of culture, cell numbers were evaluated as described in Figure 5. Each point is the average of duplicate measurements and the standard error is shown.

When tested alone, IL-6 SAnt has the same properties as the two parental mutants, i.e. a roughly 4-fold increase in binding to IL-6Rα (Table II) and no bioactivity (not shown). However, when tested for antagonism, it shows a significant improvement over DFRD (Table II), with the concentrations needed to reach 50% inhibition 8- to 10-fold lower and a significant shift in the inhibition curve (Figure 6). The super-antagonist effect was also tested on the human Epstein–Barr virus-transformed B cell line CESS, in which IL-6 induces IgG secretion (Poupart *et al.*, 1987). On CESS cells both DFRD and SAnt fully antagonize IL-6-induced IgG secretion, but SAnt is about eight times more potent. The most significant result, however, was obtained on XG-1 cells, where a 50% inhibition of cell growth is reached with a molar excess of about 200-fold (20 ng/ml) and full inhibition is obtained with concentrations of SAnt in the medium below 1 μg/ml.

Discussion

In this paper we have described the rational design of IL-6 receptor antagonists and super-antagonists which are able to fully and specifically inhibit cytokine activity in all IL-6-responsive human cell lines tested. Our results confirm and extend the initial hypothesis that it is possible to dissociate IL-6Rα binding from signal transduction through the mutagenesis of the surface area which binds to gp130 (Savino *et al.*, 1994). We identified this surface through the superposition of the IL-6 three-dimensional model onto the known structure of GH. The initial testing of the model led to the conclusion that the two systems are very similar to each other, but that subtle differences must also exist (Savino *et al.*, 1994). This suggested the further hypothesis that Site 2 in IL-6 might be shifted by 1–2 helical turns with respect to GH (see Figure 1), subsequently confirmed by the mutagenesis of residues 118 and 121 in helix C and by the combination of helix C and A mutations. This reiterative process of a molecular model that guided site-directed mutagenesis, which in turn was used to further refine the model, demonstrates that it is sometimes possible to reach a deeper understanding of a multisubunit receptor assembly even when no precise structural data is available.

Our molecular model has led to the identification of exposed residues in helices A and C as contact points with gp130, however, it does not explain recent results reported by Brakenhoff *et al.* (1994). These authors, using a different approach, have obtained a partial IL-6

antagonist by substituting residues 159 and 162, which are predicted to be located at the beginning of helix D, far from the proposed Site 2. Interestingly, the interaction of this mutant with recombinant gp130 is also strongly impaired *in vitro* (Brakenhoff *et al.*, 1994; C.Toniatti, unpublished results). Although this would apparently indicate that our model is incomplete, it might suggest the existence of a second binding site for gp130 on IL-6 (Site 3), which is perfectly in line with the observation that the last step in IL-6 receptor assembly is the homodimerization of the gp130 chain (Murakami *et al.*, 1993). More mutagenesis of IL-6 and the determination of the receptor complex stoichiometry *in vitro* will be necessary to further clarify this point.

The biochemical and biological properties of the IL-6 super-antagonist are of particular relevance. They demonstrate that Sites 1 and 2 are functionally fully independent of each other, because IL-6 SAnt maintains the characteristics of the two parental mutants IL-6/IRA and DFRD, i.e. increased binding to IL-6Rα and loss of biological activity respectively. Also, in line with the notion that IL-6Rα does not carry any relevant information for signal transduction, introducing the super-binder mutation in the context of DFRD does not increase the activity of the molecule. On the contrary, this higher receptor binding capacity causes a significant decrease in the concentration of mutant needed to inhibit wt IL-6 activity on all IL-6-responsive human cell lines tested and in particular on human XG-1 myeloma cells. In theory, the potency of the super-antagonist could be improved by further mutagenesis to progressively increase binding to IL-6Rα using an approach similar to that adopted for GH to select variants with a 400-fold increased affinity for its receptor (Lowman and Wells, 1993).

In multiple myeloma, IL-6 has been shown to function both as an autocrine and as a paracrine growth factor (Kawano *et al.*, 1988; Klein *et al.*, 1989) and to cause the development of plasmacytoma and myeloma in the kidney of transgenic mice (Suematsu *et al.*, 1989; Fattori *et al.* 1994). For these effects, generating potent antagonists of the cytokine has been anticipated as a possible therapeutic strategy. This task is made particularly arduous by the high degree of sensitivity of myeloma cells to IL-6 (see the low EC_{50} of XG-1 cells), by the high levels of cytokine produced by the majority of the patients (Lu *et al.*, 1993; Klein, 1994) and by a marginal (1.5- to 2-fold), albeit significant, increase in the circulating levels of sIL-6Rα found in patients with both early and overt myeloma (Gaillard *et al.*, 1993). An appropriate solution to this problem could be IL-6 super-antagonists, molecules that fully inhibit IL-6 at much lower dosages (in our experimental conditions below 1 μg/ml) and which therefore could be injected at therapeutically effective dosages.

Finally, the super-antagonism approach we have described for IL-6 can be applied to all other helical cytokines and growth factors (such as IL-2, IL-3, IL-4, IL-5, etc.) which drive the step-wise assembly of multisubunit receptor complexes (Kishimoto *et al.*, 1994; Wells, 1994). The work presented in this paper demonstrates that a detailed knowledge of the three-dimensional structure of the receptor complex (information not available in the case of IL-6) is not an absolute prerequisite, because structural information is often available from homologous systems, thus allowing the problem to be addressed by molecular modelling. The generation of cytokine super-antagonists is of paramount importance, given the growing interest in cytokine receptor antagonists, because it opens up new possibilities of generating proteins that can be administered at low concentrations.

Materials and methods

Generation and expression of substitution mutants

Mutagenesis at the IL-6 cDNA level, mutant protein expression in the *Escherichia coli* periplasmic space and protein quantifications were carried out as previously described (Savino *et al.*, 1994). For competition experiments, the cDNAs of the chosen mutants were subcloned in the *E.coli* expression vector pT7.7 (Studier and Moffatt, 1986) and the corresponding mutant proteins were produced in large amounts and purified as described (Arcone *et al.*, 1991).

In vitro receptor binding assays

In vitro binding assays to sIL-6Rα were performed exactly as previously described (Savino *et al.*, 1994).

Bioassays

Hep3B. Transcriptional activation of the CRP gene promoter in the human Hep3B hepatoma cell line was determined and quantified as previously described (Gregory *et al.*, 1994).

HepG2. Transcriptional activation of the CRP gene promoter in the human HepG2 hepatoma cell line was determined and quantified as previously described (Gregory *et al.*, 1994), with the only difference that 1×10^5 CRP−SEAP-transfected HepG2 cells were plated in each microtitre well, as compared with the 5×10^4 CRP−SEAP-transfected Hep3B cells (Gregory *et al.*, 1994).

A375. The effect of wt IL-6 and of IL-6 mutants, both alone and in combination, on A375 growth was determined by crystal violet staining as previously described (Horn *et al.*, 1990).

XG-1. XG-1 cells were cultured as previously described (Jourdan *et al.*, 1991). To test IL-6 mutants for biological activity and antagonistic behaviour, XG-1 cells (6×10^3/well in 96-well microtititre plates) were cultured in 200 μl of RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 5×10^{-5} M β-ME with various concentrations of each IL-6 receptor antagonist, in the presence or absence of 0.1 ng/ml wt IL-6. After 7 days, cell numbers were evaluated by colourimetric determination of hexosaminidase levels, according to Landegren (1984).
CESS. CESS cells, growing in 10% FCS−RPMI medium (Gibco), were seeded at 2×10^4 cells/well and incubated for 72 h with 2 ng/ml wt IL-6 and increasing concentrations of DFRD and SAnt. The supernatant was collected and IL-6-induced IgG1 production was measured in a sandwich ELISA using an immunopure goat anti-human IgG (Fc) serum (Pierce, Rockford, IL) to coat a microtitre plate and a goat anti-human IgG alkaline phosphatase-conjugated serum (Sigma, St Louis, MO) to reveal the amount of IgG captured by the plate. A standard curve of IgG concentration at each point was determined using a human serum (D.S.).

APRF induction and whole cell extracts preparation

Cells were induced in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS for 15 min at 37°C with various cytokine combinations. After treatment, the cultures were placed on ice, rinsed with ice-cold phosphate-buffered saline (PBS) containing 5 mM NaF and scraped from the dishes in PBS with 5 mM NaF. The cells were sedimented and cell pellets were frozen in liquid N_2. After freezing, whole cell extracts were prepared as follows: cells were lysed in 5 packed cell volumes of extraction buffer (10 mM HEPES, pH 7.8, 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 5% glycerol), lysates were ultracentrifuged at 100 000 *g* for 5 min and the cleared lysate was used for electromobility shift assay (EMSA).

Electromobility shift assays

Hep3B. APRF activation in Hep3B cells was monitored by binding to the high-affinity SIE m67 oligonucleotide (Wagner *et al.*, 1990), which was labelled by filling in 5′ protruding ends with the Klenow enzyme, using [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol). EMSA were performed essentially as described by Sadowski and Gilman (1993).

HepG2. APRF activation in HepG2 cells was monitored by binding to the α2M APRE (Wegenka *et al.*, 1993), which was labelled as described above. EMSA were performed with 5 μg whole cell extract as described by Wegenka *et al.* (1993). Complexes were resolved on 5% polyacrylamide−7.5% glycerol−0.25× TAE (1× TAE: 40 mM Tris, pH 7.8, 37 mM sodium acetate, 1.1 mM EDTA) gels.

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