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Development of a Human Interleukin-6 Receptor Antagonist*

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Neutralizing monoclonal antibodies specific for human interleukin-6 (IL-6) bind two distinct sites on the IL-6 protein (sites I and II). Their interference with IL-6 receptor binding suggested that site I is a receptor-binding site of IL-6, whereas site II is important for signal transduction. Mutagenesis of site II could therefore result in the isolation of IL-6 receptor antagonists. To test this hypothesis, a panel of IL-6 mutant proteins was constructed that did not bind to a site II-specific monoclonal antibody. One such site II mutant protein (with double substitution of Gln-160 with Glu and Thr-163 with Pro) was found to be an antagonist of human IL-6. It was inactive on human CESS cells, weakly active on human HepG2 cells, but active on mouse B9 cells. It could specifically antagonize the activity of wild-type IL-6 on CESS and HepG2 cells. The binding affinity of this variant for the 80-kDa IL-6 receptor was similar to that of wild-type IL-6. High affinity binding to CESS cells, however, was abolished, suggesting that the mutant protein is inactive because the complex of the 80-kDa IL-6 receptor and the mutant protein cannot associate with the signal transducer gp130. The human IL-6 antagonist protein may be potentially useful as a therapeutic agent.

Interleukin-6 (IL-6)[1] is a multifunctional cytokine playing a central role in host defense mechanisms (for reviews, see Refs. 1–3). IL-6 exerts its multiple activities through interaction with specific receptors on the surface of target cells (4, 5). The cDNAs for two receptor chains have been cloned and code for transmembrane glycoproteins of 80 and 130 kDa (gp130) (6, 7), which both belong to the large cytokine receptor superfamily (8–10). The 80-kDa IL-6 receptor (IL-6R) binds IL-6 with low affinity (K_d ~ 1 nM) without triggering a signal (11). The IL-6·80-kDa IL-6R complex subsequently associates with gp130, which then transduces the signal (7, 11). gp130 itself has no affinity for IL-6 in solution, but stabilizes the IL-6·80-kDa IL-6R complex on the membrane, resulting in high affinity binding of IL-6 (K_d ~ 10 pM) (7). Recently, it was found that gp130 is also a constituent of the receptors for leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor (for recent review, see Ref. 14; Refs. 12 and 13).

In a variety of human inflammatory, autoimmune, and neoplastic diseases, abnormal IL-6 production is observed and has been suggested to play a role in the pathogenesis (reviewed in Refs. 3 and 15–17; Ref. 18). A causative role for IL-6 in the pathogenesis of multiple myeloma was indeed demonstrated by the observation that administration of an anti-IL-6 antibody to a patient with plasma cell leukemia could block myeloma cell proliferation in the bone marrow (19). Thus, inhibitors of IL-6 biological activity are useful to study its role in disease and could have broad therapeutic applications.

One strategy to neutralize IL-6 activity could be by inhibition of the ligand/receptor interaction with specific receptor antagonists. The feasibility of such an approach was recently demonstrated with a natural occurring receptor antagonist for interleukin-1 (for review, see Ref. 21; Ref. 20), which is currently being tested for utility in a variety of diseases (22). For IL-6, however, no natural receptor antagonist has been identified so far.

Previously, we have shown that neutralizing monoclonal antibodies (mAbs) to human IL-6 (hIL-6) recognize two distinct epitopes, designated sites I and II, on the hIL-6 molecule (23). We speculated that one of these sites might be involved in binding to the 80-kDa IL-6R, whereas the other might be involved in an interaction with gp130. According to the IL-6R model, IL-6 variants that bind normally to the 80-kDa IL-6R, but are somehow defective in inducing the interaction of the IL-6·80-kDa IL-6R complex with gp130, might be able to prevent heterodimerization of the receptor and function as receptor antagonists.

In this paper, we investigated the roles of sites I and II in IL-6/receptor interaction. We now show evidence for a role of site I in binding to the 80-kDa IL-6R and for site II in signal transduction because a biologically inactive hIL-6 mutant protein with mutations in site II could bind to the 80-kDa hIL-6R, but antagonized the biological activity of wild-type hIL-6. Binding experiments suggest that signal transduction cannot occur because the complex of the mutant protein and the 80-kDa IL-6R cannot associate with gp130. This is the first demonstration that it is possible to separate receptor binding from biological activity of hIL-6.

MATERIALS AND METHODS

Antibodies and Cytokines

The production and purification of the IL-6-specific mAbs have been described in detail (23). mAb B1 (LN1-73-10) was a kind gift of Dr. F. Di Padova (Sandoz Pharma, Preclinical Research, Basel, Switzerland). The purified wild-type rhIL-6 preparation used throughout these experiments as a standard is derived from *Escherichia coli* carrying the HGF7 plasmid (24). Purification of rhIL-6.HGF7 has been described (23). The specific activity of purified rhIL-6.HGF7 as determined in the mouse B9 assay is ~10^9 units/mg. Recombinant IFN-γ was a kind gift from Genentech (San Francisco).

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[1] The abbreviations used are: IL-6, interleukin-6 (prefix "rh" indicates recombinant human); IL-6R, interleukin-6 receptor; mAb, monoclonal antibody; IFN-γ, interferon-γ; CV, coefficient of variation; GH, growth hormone.

Expression Vectors and Bacterial Strains

Construction of the expression vector pUK-IL-6 has been described (25). For expression of rhIL-6 or rhIL-6 mutant proteins with this vector, *E. coli* DH5α (Life Technologies, Inc.) was used as the host. The bacteriophage T7 promoter vector pET8c and expression strain *E. coli* BL21(DE3) (26) were a kind gift of Dr. G. Pruyn (Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands).

Expression Library Construction and Recombinant DNA and Sequencing Protocols

The vector pUK-IL-6 was used for construction of the library of rhIL-6 mutant proteins with randomly distributed substitutions of Gln-153–Thr-163. Construction of the library has been described in detail (27). Following transformation to *E. coli* DH5α, ~1000 colonies were obtained. DNA manipulation procedures were performed as described (23, 25). Nucleotide sequences of selected mutants (see below) were obtained with cDNA-derived oligonucleotide primers on double-stranded DNA by using the Sequenase kit (United States Biochemical Corp.).

Preparation of E. coli Extracts for Library Screening with mAbs

Four-hundred ampicillin-resistant colonies from the expression library were transferred to wells of 96-well flat-bottom microtiter plates (Nunc) containing 100 µl of LC amp medium (10 g of Bacto-Tryptone, 5 g of yeast extract, 8 g of NaCl, 2 ml of Tris base/liter supplemented with 100 µg/ml ampicillin (final concentration)). Following overnight culture at 37 °C, bacteria were lysed by addition of lysozyme to 1 mg/ml and further incubation for 30 min at 37 °C. One in 10 dilutions of the crude extracts in phosphate-buffered saline, 0.02% Tween 20, 0.2% gelatin was directly tested for reactivity in sandwich enzyme-linked immunosorbent assays with site I-specific mAb CLB.IL-6/8 (mAb8(I)) or site II-specific mAb CLB.IL-6/16 (mAb16(II)) coated on the plastic and biotinylated polyclonal goat anti-rhIL-6 as detecting antibody as described (23, 27). The nucleotide sequences were determined for mutant proteins that bound to mAb8(I), but not to mAb16(II).

Preparation and Quantification of E. coli Extracts for Biological Activity Measurements

To measure the biological activity of the mutant proteins that bound to mAb8(I), but not to mAb16(II), overnight cultures of *E. coli* DH5α carrying the mutant constructs were diluted 1:50 in 250 ml of LC amp medium and subsequently cultured to an absorbance at 550 nm of 1.5. Bacteria were harvested by centrifugation, resuspended in 5 ml of lysis buffer (phosphate-buffered saline, 1% Tween 20, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride), and lysed by sonication. To solubilize rhIL-6-containing inclusion bodies, SDS was subsequently added to 1%. After 1 h of incubation at room temperature, SDS-insoluble material was removed by centrifugation (13,000 × *g* for 15 min). The biological activity of this SDS-solubilized material was directly measured in the mouse B9 and CESS assays starting from a 1:1000 dilution. At this dilution, the SDS did not affect the bioassays used. The IL-6 variant protein concentration of these preparations was determined by a competitive inhibition radioimmunoassay with IL-6-specific mAb CLB.IL-6/7 coupled to Sepharose 4B (Pharmacia LKB, Uppsala) and ^{125}I-rhIL-6 in the presence of 0.1% SDS. Unlabeled rhIL-6 served as a standard. mAb CLB.IL-6/7 binds heat- and SDS-denatured IL-6 and recognizes IL-6 residues Thr-143–Ala-146 as determined by pepscan analysis (28, 29).[2]

Expression and Purification of Mutant Proteins T163P and Q160E,T163P from E. coli

The IL-6 cDNA inserts from the vectors pUK-IL-6 T163P and pUK-IL-6 Q160E,T163P were subcloned in the vector pET8c, and the plasmids were transformed to *E. coli* BL21(DE3) for expression (26). The rhIL-6 variants were subsequently purified essentially as described (30). Briefly, the proteins were prepared from inclusion bodies by extraction with 6 M guanidine HCl, renaturation by dialysis against 25 mM Tris (pH 8.5), and purification by anion-exchange chromatography. The preparations were free of contaminating *E. coli*-derived proteins as judged by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue or silver staining. In some of the preparations, two bands were observed corresponding to the full-length mature protein and an ~10-amino acid shorter IL-6-derived degradation product. This degradation product does not significantly change the biological activity or the receptor binding of the full-length product because it generally constituted only 10–20% of the preparations. Moreover, carboxyl-terminal cleavage of only 5 amino acids of IL-6 reduces both the bioactivity and receptor binding affinity 1000-fold (23),[3] and amino-terminal cleavage of ~10 amino acids results in a product that behaves very similar to the wild-type molecule (25). Protein concentration was determined both by measuring the absorbance of the preparations and by the Bradford method (64) using bovine serum albumin as a standard. Bradford and $A_{280\ nm}$ data correlated best when assuming that the absorbance at 280 nm of a 10 mg/ml solution of IL-6 is 10.

IL-6 Bioassays

Mouse B9 Assay—The hybridoma growth factor activity of rhIL-6 and variants was measured in the mouse B9 assay as described (31).

CESS Assay—B cell stimulatory factor-2 activity of rhIL-6 variants was measured as described (32). IL-6-induced IgG1 production by the cells was subsequently measured in a sandwich enzyme-linked immunosorbent assay using a mouse mAb specific for human IgG1 (MH161-1M; from the Department of Immune Reagents, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (C.L.B.), Amsterdam) in combination with a horse radish peroxidase-conjugated mouse mAb specific for human IgG (MH16-1ME; C.L.B.) with a human serum as a standard (H00-1234; C.L.B.). Enzyme-linked immunosorbent assay procedures were as described above.

HepG2 Assay—The hepatocyte stimulating activity of rhIL-6 variants was assessed by measuring the induction of C1 esterase inhibitor production by HepG2 cells as described (33). Following culturing to confluency (5×10^5 cells in 0.5-ml wells (Costar) in Iscove's modified Dulbecco's medium supplemented with 5% fetal calf serum, 5×10^{-5} M β-mercaptoethanol, 100 IU of penicillin, 100 µg/ml streptomycin, and 20 µg/ml human transferrin (Behringwerke, Marburg, Germany), HepG2 cells were washed twice and stimulated with serial dilutions of rhIL-6 or rhIL-6 mutant proteins for 48 h in the same medium in duplicate. In some experiments, cells were washed again after 24 h, and the cultures were continued for another 24 h in the presence of the same stimulus. This procedure results in a higher stimulation index. After the incubation period, C1 esterase inhibitor synthesis was subsequently measured by sandwich radioimmunoassay with anti-C1 esterase inhibitor mAb RII coupled to Sepharose 4B and ^{125}I-labeled sheep polyclonal anti-C1 esterase inhibitor IgG with normal human plasma as a standard as described (34).

Binding Experiments

The inhibitory effect of the mAbs on IL-6 binding to CESS cells was measured by using metabolically ^{35}S-labeled rhIL-6 as described (5, 35). The inhibitory effect of rhIL-6 and of the rhIL-6 Q160E,T163P mutant protein on ^{125}I-rhIL-6 binding to NIH-3T3 fibroblasts transfected with an 80-kDa IL-6R expression vector was measured as described (36). Inhibition of binding of ^{125}I-IL-6 to the soluble IL-6R was measured as follows. The extracellular ligand-binding domain of the 80-kDa IL-6R was expressed in NIH-3T3 fibroblasts as described (37). Culture supernatants of these cells were diluted 1:2 in 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM methionine, 0.01% NaN_3 and incubated with 5×10^4 dpm ^{125}I-IL-6 in the absence or presence of increasing concentrations of the rhIL-6 or Q160E,T163P protein for 2 h at 4 °C. ^{125}I-IL-6-soluble hIL-6R complexes were immunoprecipitated using an 80-kDa receptor-specific antiserum and protein A-Sepharose. Sepharose-bound radioactivity was subsequently measured with a γ-counter (37).

For Scatchard analysis of wild-type rhIL-6 and Q160E,T163P binding to CESS cells, rhIL-6 purified from Chinese hamster ovary cells expressing hIL-6 (a kind gift from Dr. D. Fischer, Interpharm Laboratories, Nes-Ziona, Israel) and Q160E,T163P, purified as described above, were labeled with ^{125}I-Bolton-Hunter reagent (4000 Ci/mmol, diiodinated; Amersham, Amersham, United Kingdom) essentially as described (4, 38). Briefly, 5 µg of rhIL-6 or mutant in 50 µl of borate buffer (50 mM $NaBO_3$, 0.02% Tween 20 (pH 8.5)) was added to 500 µCi of dried Bolton-Hunter reagent. rhIL-6 was incubated for 30 min at room temperature, and Q160E,T163P for 15 min at 0 °C with occasional mixing. Both reactions were stopped by addition of 100 µl of 5 mg/ml glycine in phosphate-buffered saline for 10 min. ^{125}I-Labeled rhIL-6 or

[2] J. P. J. Brakenhoff, M. Hart, F. Di Padova, and L. A. Aarden, manuscript in preparation.

[3] J. P. J. Brakenhoff, F. D. de Hon, V. Fontaine, E. ten Boekel, H. Schooltink, S. Rose-John, P. C. Heinrich, J. Content, and L. A. Aarden, unpublished results.

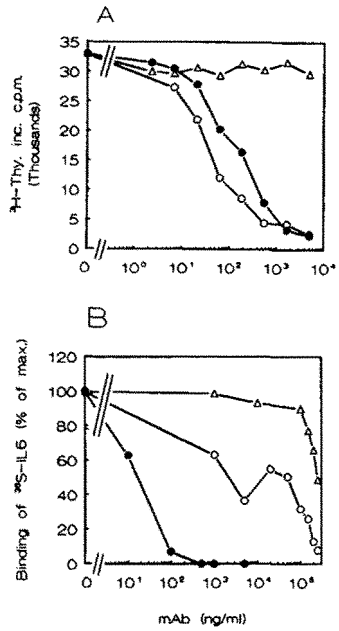

FIG. 1. **Differential effects of mAbB1(I) and mAb16(II) on IL-6 biological activity and receptor binding.** *A*, inhibition of mouse B9 hybridoma proliferation induced by 2 units/ml human monocyte-derived IL-6 with increasing concentrations of mAbB1(I) (●), mAb16(II) (○), and mAb14(III) (△). Data represent the mean of triplicate measurements. *B*, inhibition of ^{35}S-IL-6 binding to CESS cells. CESS cells were incubated with 80 pM ^{35}S-IL-6 in the absence or presence of increasing concentrations of the mAbs or unlabeled IL-6, and the amount of specifically bound IL-6 was measured as described under "Materials and Methods." Data represent the mean of duplicate measurements and are expressed as percent of maximum specific bound IL-6.

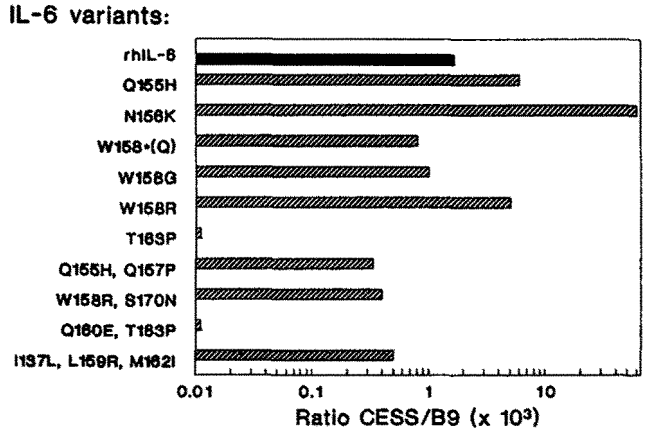

FIG. 2. **Biological activity of hIL-6 mutant proteins that do not bind to mAb16(II).** The hIL-6 site II mutants were selected with mAb8(I) and mAb16(II) from a hIL-6 expression library in *E. coli* as described in the text and under "Materials and Methods." The specific activity in mouse B9 and CESS assays was measured in SDS extracts of *E. coli* carrying plasmids encoding the rhIL-6 site II mutant proteins as described under "Materials and Methods." Data are expressed as the ratio of specific activity (units/microgram) in the CESS and mouse B9 assays. Values are derived from one of two experiments. *rhIL-6* indicates mature rhIL-6, and the *asterisk* in *W158*(Q)* indicates the TAG stop codon (due to the supE44 mutation in *E. coli* DH5α, a glutamine is incorporated in a fraction of the protein).

mutant was separated from ^{125}I-labeled products of low molecular mass by gel filtration chromatography on a PD-10 column (Pharmacia), sterile-filtered, and stored at 4 °C. The specific radioactivity of the preparations was determined by self-displacement analysis and was corrected for maximal binding capacity as described by Calvo *et al.* (39). The maximal binding capacity was 38% for ^{125}I-rhIL-6 and 16% for ^{125}I-Q160E,T163P. The specific activity of both preparations was ~160 Ci/mmol.

Binding assays were performed as described by Shanafelt *et al.* (40). Briefly, CESS cells were harvested, washed once, and resuspended in binding buffer (RPMI 1640 medium, 10% fetal calf serum, 50 mM Hepes, 0.02% NaN_3 (pH 7.4)). 2 × 10^6 viable cells/point were incubated with decreasing concentrations of ^{125}I-rhIL-6 or ^{125}I-Q160E,T163P in triplicate at 4 °C with continuous agitation for 3 h. Nonspecific binding was determined by including unlabeled rhIL-6 or Q160E,T163P as appropriate at a 500-fold excess. Cell-bound radioactivity was separated from free radioactivity by oil centrifugation, and bound and total radioactivities were counted with a Cobra 5010 γ-counter (40). The standard deviation of the triplicates was generally <5%. The equilibrium binding data were analyzed using the LIGAND program (41).

RESULTS

Site II mAb That Strongly Inhibits Biological Activity Has Little Effect on Receptor Binding of IL-6—The inhibitory effect of site I- and II-specific mAbs (23) was compared both in IL-6 bioassays and in IL-6 receptor binding experiments. Fig. 1*A* demonstrates the inhibitory effects of site I-specific mAb LN1-73-10 (mAbB1(I)) and site II-specific mAb CLB.IL-6/16 (mAb16(II)) on IL-6 biological activity in the mouse B9 hybridoma proliferation assay. The order of potency in this experiment was mAb16(II) > mAbB1(I), with EC_{50} values of ~40 and 180 ng/ml, respectively. A non-neutralizing control mAb (site III-specific mAb CLB.IL-6/14, mAb14(III) (23)) had no effect up to 5 μg/ml. This relative potency of the mAbs was observed in a number of experiments and was similar when tested on human cells.

To compare the inhibitory effects of the site I- and II-specific mAbs on biological activity with that on receptor binding of IL-6, human CESS cells (an Epstein-Barr virus-transformed B cell line) were incubated with ^{35}S-rhIL-6 in the presence of increasing concentrations of the mAbs. Fig. 1*B* shows that mAbB1(I) completely inhibited IL-6R binding at a concentration of 500 ng/ml, whereas mAb16(II) was unable to fully inhibit rhIL-6 binding even at a concentration of 250 μg/ml. At this concentration, the control mAb, mAb14(III), also inhibited receptor binding. So, whereas mAb16(II) inhibited biological activity of IL-6 more efficiently than mAbB1(I), also when tested with ^{35}S-rhIL-6 used in Fig. 1*B* (data not shown), it hardly interfered with the binding of IL-6 to CESS cells.

Construction and Analysis of Expression Library of Site II Mutant Proteins of IL-6—There are multiple ways in which mAb16(II) could inhibit biological activity without preventing receptor binding of IL-6. One of the possibilities was that site II, the epitope recognized by mAb16, is directly involved in signal transduction of IL-6 through interaction with gp130. Mutagenesis in this region could therefore result in the isolation of IL-6 mutant proteins defective in signal transduction, but not in receptor binding. In previous experiments, we had observed that binding of mAb16(II) to rhIL-6 was abolished by substitution of Trp-158 (27). However, this substitution protein was fully active when tested in the mouse B9 assay. Random mutagenesis of the region around Trp-158 was therefore performed to identify other residues involved in mAb16(II) binding, to perhaps uncover some that are also important to the biological activity of IL-6. A library of plasmids encoding rhIL-6 mutant proteins with randomly distributed substitutions of Gln-153–Thr-163 was constructed in the *E. coli* expression vector pUK-IL-6 (25). To select for variants with an intact receptor-binding site, the library was screened for mutant proteins that bound to a site I-specific mAb, but not to site II-specific mAb16. The nucleotide sequences of plasmids encoding mutant proteins with this phenotype were subsequently determined. The phenotype and the biological activity of the mutants are depicted in Fig. 2. Single substitutions of Gln-155, Asn-156, Trp-158, and Thr-163 disrupted the mAb16(II) epitope. From the double- and triple-substitution mutant proteins, it can be concluded that Leu-159, Gln-160, and Met-162 might also be im-

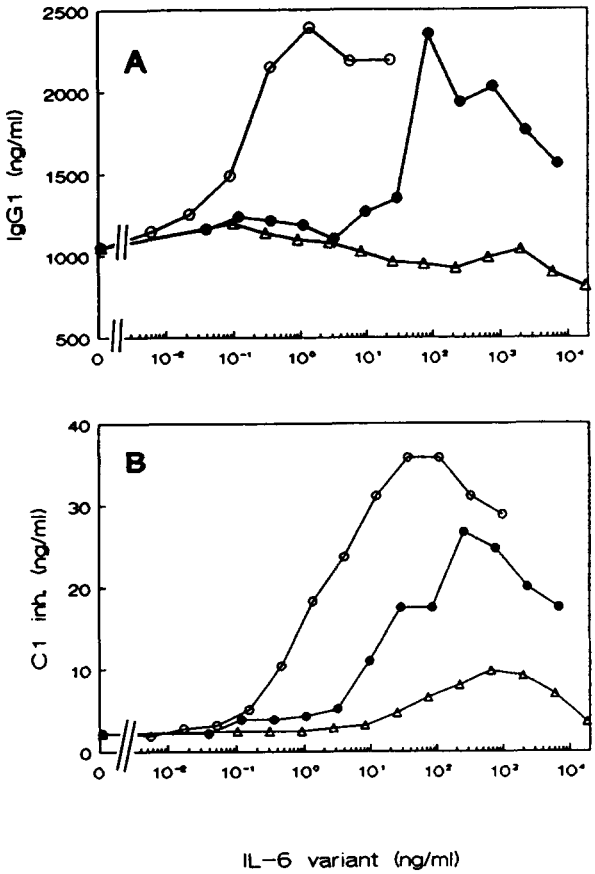

FIG. 3. **Activity of purified rhIL-6 variants in different bioassays for human IL-6.** Shown is induction by wild-type rhIL-6 (○), T163P (●), and Q160E,T163P (△) of IgG1 production by CESS cells (*A*) and C1 esterase inhibitor (*C1 inh.*) production by HepG2 cells (*B*). For each assay, one representative experiment is shown. Data points represent averages of three measurements. Assays were performed as described under "Materials and Methods."

portant for the mAb16 epitope, but this has yet to be confirmed by analyzing mutant proteins with single substitutions of these residues.

Some Site II Mutant Proteins Are Inactive on Human CESS Cells—The biological activity of crude extracts of the various mutant proteins was subsequently measured both in the mouse B9 assay and in IgG1 production by human CESS cells. All mutant proteins were biologically active in the mouse B9 assay. However, although very active in the mouse B9 assay, no activity could be detected in the rhIL-6.T163P and rhIL-6.Q160E,T163P mutant protein preparations on CESS cells (Fig. 2).

Selective Agonism of Q160E,T163P on Human Cells—To confirm the above observation, both IL-6 mutant proteins were purified and tested for biological activity on CESS cells and on a second IL-6-responsive cell line of human origin. Fig. 3 (*A* and *B*) shows representative dose-response curves of the mutant proteins in the two assays. In Table I, the specific activities of the mutant proteins in these assays are depicted, together with the specific activities in the mouse B9 assay. The T163P mutant protein was active in all assays, but with a lower specific activity than that of wild-type rhIL-6. As with the crude protein, the purified Q160E,T163P mutant protein again did not induce IgG1 synthesis by the CESS cells, not even when tested at a 10^5-fold higher concentration than the wild-type protein (Fig. 3*A*). At these high concentrations, this variant protein caused a weak increase in the production of the acute-phase protein C1 esterase inhibitor by HepG2 cells. This weak response was

TABLE I
Specific activity of purified rhIL-6 variants in IL-6 bioassays

rhIL-6 variant	Bioassay[a]		
	Mouse B9	CESS	HepG2
rhIl-6.HGF7	1.7	213	1.5×10^3
T163P	5	3.3×10^5	9.1×10^4
Q160E,T163P	11	$>10^7$	$>10^7$

[a] EC_{50} values (the concentration of IL-6 variant giving a half-maximal response in the assays), expressed in picograms/milliliter, were estimated graphically from dose-response curves of the purified proteins. Values represent averages of at least three experiments performed over a time span of several months. S.D. values varied from 14 to 90% of these values.

characterized by a strongly reduced maximal response as compared to wild-type rhIL-6 (Fig. 3*B*). On mouse B9 cells, the specific activity of Q160E,T163P was 6–7-fold lower than that of rhIL-6 (Table I).

Antagonism of IL-6 Action on CESS and HepG2 Cells by Q160E,T163P—We subsequently tested whether these mutant proteins were able to antagonize the biological activity of wild-type rhIL-6. Fig. 4 (*A* and *B*) shows that the Q160E,T163P mutant protein completely inhibited the wild-type IL-6 activity on CESS and HepG2 cells. In these experiments, 50% inhibition of IL-6 activity in CESS and HepG2 assays was observed with ~50 ng/ml and 1 μg/ml Q160E,T163P respectively, corresponding to 20- and 200-fold the concentration of wild-type rhIL-6 used to stimulate the cells. No antagonistic activity of the T163P mutant protein could be detected (data not shown). The inhibitory effect of Q160E,T163P on IL-6 activity in both the CESS and HepG2 assays could be reversed by high concentrations of wild-type rhIL-6, suggesting that the inhibitory mechanism is competitive inhibition of IL-6 receptor binding by Q160E,T163P (data not shown).

Specificity of Antagonism by Q160E,T163P—The production of the acute-phase protein C1 esterase inhibitor by HepG2 cells can be increased in response to both IL-6 and IFN-γ via separate mechanisms (33). To demonstrate the specificity of inhibition by the double-mutant protein, we tested whether Q160E,T163P could inhibit IFN-γ-induced C1 esterase inhibitor synthesis by the HepG2 cells. Because IFN-γ is more potent than IL-6 in this assay, we tested the effect of Q160E,T163P over a concentration range of IFN-γ. No inhibitory effects of Q160E,T163P were observed at any IFN-γ concentration tested. An example is shown in Fig. 5, demonstrating that the C1 esterase inhibitor synthesis induced by 5 ng/ml wild-type rhIL-6 was inhibited to background levels, whereas that induced by 1 ng/ml IFN-γ was unimpaired.

Binding to 80-kDa IL-6R by Q160E,T163P—The fact that the mutant protein Q160E,T163P could still be recognized by site I-specific mAb8 and that it could antagonize wild-type IL-6 activity on CESS and HepG2 cells suggested that the 80-kDa IL-6R-binding site of the mutant protein was still intact. To test this hypothesis, binding of this variant to the 80-kDa IL-6R (to both the membrane-bound and -soluble forms) was measured. In Fig. 6*A*, the capacity of Q160E,T163P to inhibit binding of ^{125}I-IL-6 to NIH-3T3 fibroblasts transfected with an expression vector encoding the 80-kDa IL-6R (36) was compared to that of wild-type rhIL-6. Fig. 6*B* shows the effect of the mutant protein and wild-type rhIL-6 on binding of ^{125}I-IL-6 to the soluble IL-6R. The results indicate that the mutant protein Q160E,T163P was 3–4-fold less efficient than wild-type rhIL-6 in inhibiting binding of ^{125}I-rhIL-6 to the 80-kDa IL-6R.

No High Affinity Binding of Q160E,T163P to CESS Cells—The above data suggest that Q160E,T163P is inactive on human cells because, although it can efficiently bind to the 80-kDa IL-6R, the complex of mutant and receptor is deficient in triggering signal transduction through gp130. To test

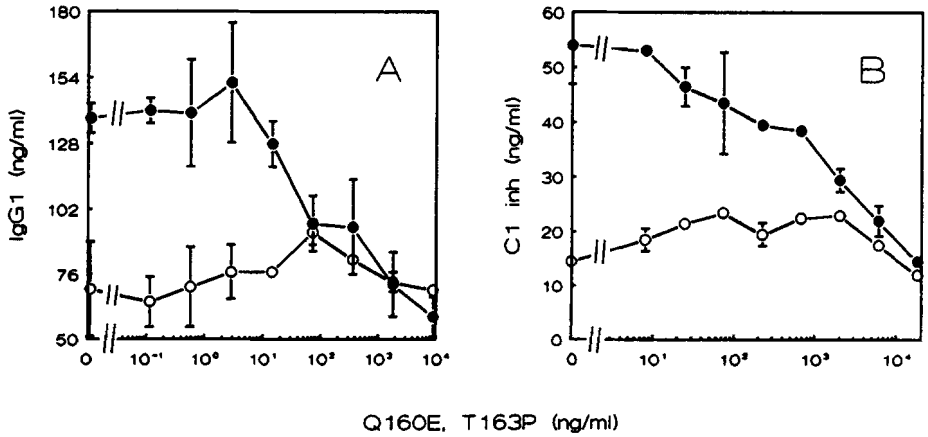

FIG. 4. **Antagonism of hIL-6 activity by Q160E,T163P.** *A*, CESS cells were incubated with increasing concentrations of Q160E,T163P in the absence (○) or presence (●) of 2 ng/ml wild-type rhIL-6. Data are expressed as means ± S.D. (n = 3). *B*, HepG2 cells were incubated for 48 h with increasing concentrations of Q160E,T163P with or without 5 ng/ml rhIL-6. Data points are means ± S.D. (n = 2). For both assays, one representative experiment of three is shown. *C1 inh.*, C1 esterase inhibitor.

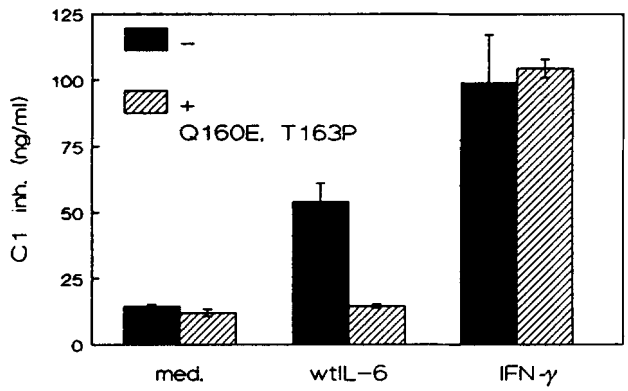

FIG. 5. **Q160E,T163P inhibits biological activity of hIL-6, but not of IFN-γ on HepG2 cells.** HepG2 cells were incubated with or without rhIL-6 (5 ng/ml) or recombinant human IFN-γ (1 ng/ml) in the absence or presence of 9 μg/ml Q160E,T163P. One of two experiments is shown. Data are means ± S.D. (n = 2). *C1 inh.*, C1 esterase inhibitor; *med.*, medium; *wt*, wild-type.

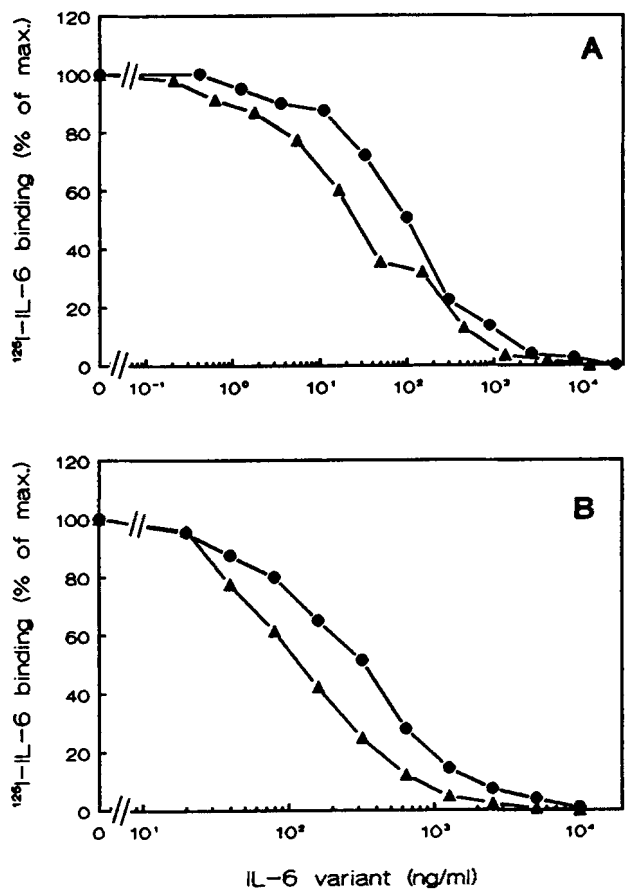

FIG. 6. **Q160E,T163P has affinity for 80-kDa IL-6R similar to that of wild-type rhIL-6.** *A*, competitive inhibition of 15 ng/ml (700 pM) ^{125}I-rhIL-6 binding to NIH-3T3 fibroblasts expressing the 80-kDa IL-6R with increasing concentrations of rhIL-6 (▲) or Q160E,T163P (●); *B*, inhibition of binding of 5 ng/ml (240 pM) ^{125}I-rhIL-6 to the soluble form of the 80-kDa hIL-6R with rhIL-6 and Q160E,T163P. Averages of duplicate measurements are shown for both assays. Assays were performed as described under "Materials and Methods."

whether Q160E,T163P could still bind with high affinity to human cells, we performed receptor binding experiments using ^{125}I-labeled Q160E,T163P and CESS cells. Coulie *et al.* (5) and Taga *et al.* (4) detected a single class of binding sites on CESS cells with K_d values of ~30 pM (1700 sites/cell) and 340 pM (2700 sites/cell), respectively. However, both with *E. coli*-derived ^{125}I-rhIL-6 (data not shown) and with Chinese hamster ovary cell-derived glycosylated rhIL-6, we always observed two binding sites. In Fig. 7, the results from representative experiments are displayed in Scatchard plots. Two binding sites were statistically significant for wild-type rhIL-6 (Fig. 7*A*; F = 26.14 (p = 0.001) for a one- *versus* two-site fit), with K_d values of 13 pM (coefficient of variation (CV) = 40%, 430 sites/cell (CV = 30%)) and 360 pM (CV = 31%, 3200 sites/cell (CV = 9%)). Q160E,T163P, however, exhibited only a single class of binding sites, with a K_d of 500 pM (Fig. 7*B*; CV = 59%, 7000 sites/cell (CV = 49%)). A two-site fit of the ^{125}I-Q160E,T163P binding data was not statistically significant (F = 0.03 (p = 0.9710) for a one- *versus* two-site fit). From this experiment, we concluded that Q160E,T163P is inactive on CESS cells because the binding of the mutant·80-kDa IL-6R complex to gp130 cannot occur.

DISCUSSION

In this paper, we show for the first time that receptor binding and signal transduction of hIL-6 can be uncoupled. This is evidenced by the following observations. 1) The Q160E,T163P mutant protein was inactive on CESS cells (the responses of Q160E,T163P are within the standard deviation of the background control), yet antagonized the biological activity of wild-type rhIL-6 on these cells. 2) The antagonist protein had an affinity similar to that of wild-type rhIL-6 for the 80-kDa IL-6R. According to the model for IL-6/receptor interaction, high

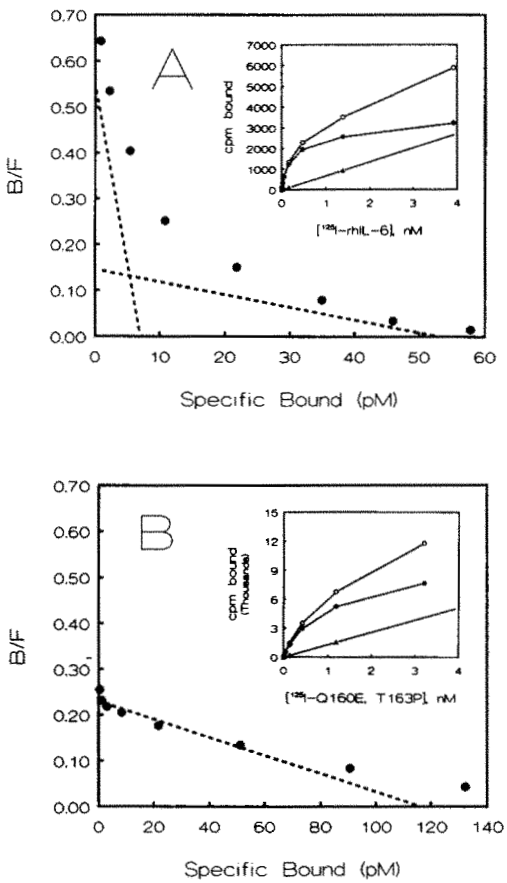

FIG. 7. **Scatchard analysis of rhIL-6 and Q160E,T163P binding to CESS cells.** Scatchard transformations of the binding of ^{125}I-rhIL-6 (*A*) and ^{125}I-Q160E,T163P (*B*) are shown. Data are derived from representative experiments with the same batch of CESS cells. Similar results were obtained in three independent experiments. *Dashed lines* represent the best fit values obtained from the LIGAND program (41) for these experiments. Wild-type rhIL-6 showed both high and low affinity binding (K_d = 13 pM (430 sites/cell) and 360 pM (3200 sites/cell); F = 26.14 (p = 0.001) for a two- *versus* one-site fit). Q160E,T163P exhibited a single class of binding sites, with a K_d of 500 pM (7000 sites/cell; a two-site fit was not statistically better than a one-site fit (F = 0.03 (p = 0.9710)). *Insets*, equilibrium binding isotherms (total bound (○), specific bound (●), and nonspecific bound (▲) ^{125}I-rhIL-6 or ^{125}I-Q160E,T163P) for the respective Scatchard plots are shown. B/F, bound/free.

affinity binding of the IL-6·80-kDa IL-6R complex to gp130 is a prerequisite for IL-6 signal transduction (7, 11). Our observations support this model because ^{125}I-Q160E,T163P bound only with low affinity to CESS cells, suggesting that the complex of the mutant protein and the 80-kDa IL-6R could not associate with gp130 to trigger a signal. Because IL-6 does not bind to gp130 in the absence of the 80-kDa IL-6R, it is not clear whether there is a direct interaction between IL-6 and gp130 in the complete receptor complex (11). Recent evidence from cross-linking experiments suggests that IL-6 and gp130 are in very close proximity in the IL-6·receptor complex (42, 43). Without structural information from crystallographic studies on the ligand-receptor complex, however, it is difficult to prove the existence of a contact and the possible role of IL-6 residues Gln-160 and/or Thr-163 therein.

Selective Agonism of Q160E,T163P—On HepG2 cells, at high concentrations of the Q160E,T163P mutant protein, we reproducibly observed a small partial agonist activity, characterized by a maximal response of ~10–20% of that of wild-type rhIL-6 (Fig. 3*B*). This might indicate that the antagonist·80-kDa IL-6R complex still exhibits some affinity for gp130, which we did not detect in the binding experiments with CESS cells. For the recently described hIL-4 (44) and mouse IL-2 (45) antagonist variants, the response also varied between cell lines studied and could be explained by differences in sensitivity of the cell lines to the respective wild-type cytokines: in the more sensitive cell lines, full receptor occupancy was not required to elicit a maximal response. Partial agonist activity of a mutant that was inactive on insensitive cells was explained by occupancy of spare functional receptors on the very responsive cell types, compensating for the receptor activation defects of the mutants (44, 45). This explanation cannot be applied to our results, however, because CESS cells, which were nonresponsive to the Q160E,T163P mutant, were more sensitive to wild-type rhIL-6 than HepG2 cells: for HepG2 cells, half-maximal stimulation was achieved at 70 pM rhIL-6, whereas 10 pM induced half-maximal stimulation of CESS cells (Table I). Although we have as yet no explanation for this observation, our results might be due to differences in IL-6 receptors on CESS and HepG2 cells. Pietzko *et al.* (43) recently showed that the gp130 molecule on HepG2 cells seems to differ in molecular mass from that on leukocytes and postulated the existence of a third receptor chain necessary for high affinity binding, in analogy with the IL-2 system (46). Furthermore, there might be differences in the relative numbers of high and low affinity receptors on CESS and HepG2 cells (Fig. 7*A*) (43).

In contrast to its activity on human cells, the Q160E,T163P mutant protein was nearly fully active on mouse cells, with a 6–7-fold reduced specific activity as compared to wild-type rhIL-6 in the mouse B9 assay. At first glance, this seemed to be due to the extremely high sensitivity of B9 cells to IL-6: half-maximal proliferation is induced by 0.08 pM, corresponding to a receptor occupancy of only 0.8%, assuming a high affinity K_d for the mouse IL-6R of ~10 pM. However, when we tested the Q160E,T163P mutant protein on the insensitive mouse plasmacytoma cell line T1165, which requires 10 pM IL-6 for half-maximal activation, as with B9 cells, the specific activity was ~15% of that of wild-type rhIL-6. This suggests that in the mouse system, the interaction of the Q160E,T163P·80-kDa IL-6R complex with gp130 is not as strongly affected as in the human system and that the double mutant should still be able to bind with high affinity to mouse cells. Further experiments are in progress to resolve the above issues.

Localization of Gln-160 and Thr-163 in Putative Tertiary Structure of hIL-6—Unfortunately, the tertiary structure of IL-6 is unknown. Based on homology comparisons, however, IL-6 belongs to the large group of cytokines that have an antiparallel four-α-helical bundle core structure similar to that of growth hormone, which include granulocyte/colony-stimulating factor, myelomonocytic growth factor, erythropoietin, and prolactin and also oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor (8, 47, 48). For IL-6, the GH structure indeed seems to be a useful working model: Fig. 8 shows the localization of sites I and II in the hypothetical three-dimensional model for hIL-6 based on the GH structure. mAbB1(I) was shown to recognize residues at both the amino and carboxyl termini of IL-6, which fits with close proximity of helices A and D in the model (23). Site I and II-specific mAbs are capable of forming a sandwich with monomer rhIL-6 in enzyme-linked immunosorbent assays, which also agrees with the model (23). Site I on IL-6 and residues close to it are likely to be the 80-kDa receptor-binding site. 1) Site I-specific mAbs strongly inhibit receptor binding, and 2) site I colocalizes with Ser-178–Arg-185, which were recently shown to be essential for biological activity and receptor binding of IL-6 (23, 49–53). Site I also maps closely to a region essential to bioactivity in the NH_2-terminal α-helix (Ile-30–Asp-35) (25, 29). This region on hIL-6 partially colocalizes with binding site 1 on human GH,

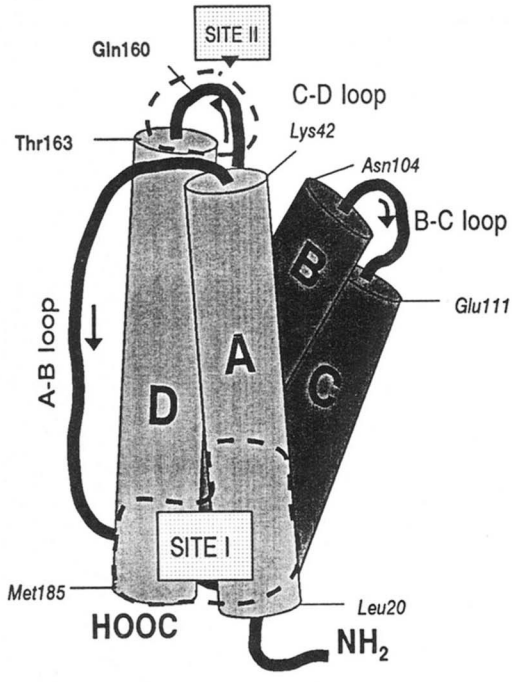

FIG. 8. **Locations of sites I and II, Gln-160, and Thr-163 in putative tertiary structure of hIL-6.** The structure is based on that of GH (62, 63) and is adapted from Ref. 8. Only the helix bundle core and loop connectivity are shown. IL-6 residues predicted to be at the end of the α-helices are derived from Ref. 47. *Helix A*, Leu-20–Lys-42; *helix B*, Glu-82–Asn-104; *helix C*, Glu-111–Lys-132; *helix D*, Thr-163–Met-185. Localization of site I is derived from Ref. 23, and that of site II from Fig. 2.

which was identified as a patch consisting of three discontinuous segments: the loop between GH residues 54 and 74 (the A-B loop), the COOH-terminal half of helix D, and, to a lesser extent, the NH_2-terminal region of helix A (54, 55). Whether the A-B loop in hIL-6 is also part of the 80-kDa binding site is as yet unknown.

Gln-160 and Thr-163 are located in the C-D loop and at the beginning of helix D, respectively, and are part of site II. This region does not colocalize with site 2 on human GH, which consists of residues near the NH_2 terminus, and on the hydrophilic faces of helices A and C (56). It does colocalize, however, with one of the regions of greatest similarity between neuropoietic and hematopoietic cytokines (including IL-6, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor), which was called the "D1 motif" (47). Because gp130 is part of the receptor complex of IL-6 (12, 13) and is essential for signal transduction by IL-6, ciliary neurotrophic factor, leukemia inhibitory factor, and oncostatin M (57, 58), it would be interesting to test the effect of mutations in the corresponding D1 regions of these cytokines on biological activity and gp130 interaction.

Conclusion—In this report, we described for the first time the isolation of an IL-6 analog that could antagonize the activity of wild-type hIL-6 in some *in vitro* bioassays. The dose range in which this molecule inhibited IL-6 activities in these bioassays is similar to that reported for the natural occurring IL-1ra (59). The pleiotropy of IL-6 and the broad distribution of its receptors make selective therapeutical application of receptor antagonists difficult to envision. However, the observation that it is possible to isolate an IL-6 variant that is active in some (but not all) bioassays may point to the possibility of isolating selective agonists of IL-6 that retain useful activities, but antagonize IL-6 activity where desired. Otherwise, combination of an IL-6 antagonist with other cytokines that share some (but not all) of the functions of IL-6 might in part restore the useful activities of IL-6. Detailed information concerning the *in vivo* sites of production and the activities of IL-6 and cytokines with similar activities is required before considering these options. Development of IL-6 inhibitor strategies seems very appropriate regarding the speed with which diseases are discovered in which IL-6 seems to play a role (60, 61).

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