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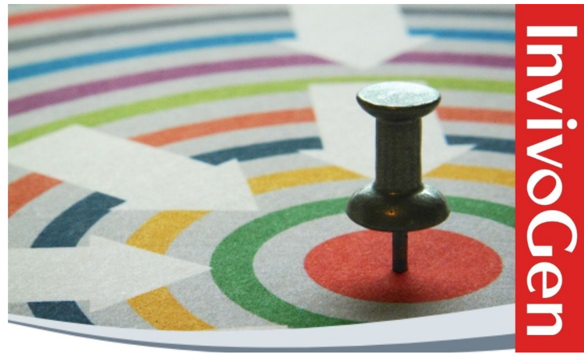

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EFFECTS OF SITE-SPECIFIC MUTATIONS ON BIOLOGIC ACTIVITIES OF RECOMBINANT HUMAN IL-6[1]

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To examine structure-activity relationships of human IL-6, we have determined the effects of specific mutations on the biologic activity of a human rIL-6 expressed in bacteria. Three types of mutants were examined: 1) a variant that contains serines in place of the four naturally occurring cysteines; 2) a series of cysteine-containing deletion mutants, each having a single internal 20 amino acid deletion; and 3) a cysteine-free variant containing a single 20 amino acid deletion. The mutants of the second type constitute a set of nonoverlapping, adjacent deletions spanning amino acids 4 through 183 of the 184 amino acids in natural human IL-6. All of the mutants were expressed, along with the full length, cysteine-containing analogue, in *Escherichia coli* as fusion proteins, joined to β-galactosidase through a collagen linker. This system allows microgram quantities of the rIL-6 variants to be partially purified from small bacterial cultures without chromatographic or refolding steps. Each of the rIL-6 variants was released from the β-galactosidase fusion protein with collagenase, and the recovered rIL-6 was quantitated by laser densitometry of Coomassie-stained, SDS polyacrylamide gels. The sp. ac. of each of the rIL-6 variants was determined using four assays: induction of IgM secretion from an EBV transformed human B cell line, induction of fibrinogen secretion from a human hepatoma cell line, induction of fibrinogen secretion from a rat hepatoma cell line, and induction of proliferation of a murine hybridoma cell line. Replacement of cysteines with serines reduced activity relative to cysteine-containing rIL-6 to about 20% in the rat hepatoma assay and about 3% in the mouse hybridoma assay, whereas activity in both of the human cell lines was reduced to less than 0.1%. These data suggest that the murine and rat cell lines are less selective than the human cell lines in their requirements for recognition of biologically active IL-6. Each of the deletions, except that of amino acids 4 through 23, resulted in loss of activity in all four assays. These results suggest that the information necessary for activity is not contained within any one portion of the IL-6 molecule, but rather that multiple segments of the protein are required for each of the biologic activities that we tested.

Human IL-6 is a 184 amino acid cytokine secreted by a number of cell types including monocytes, T cells, fibroblasts, keratinocytes, and endothelial cells (1–5). Biologically active IL-6 has been successfully expressed in transfected mammalian cell lines, injected *Xenopus* oocytes, cell-free reticulocyte lysate, insect cells, and *Escherichia coli* (2, 6–9). The biologic activities demonstrated for IL-6 are numerous and include induction of final maturation of B cells (10), stimulation of growth and differentiation of T cells (11, 12), regulation of acute phase protein production in hepatocytes (1), stimulation of proliferation of hybridoma/plasmacytoma/myeloma cell lines (13), and multiple CSF activities on hemopoeitic stem cells (14). Receptors specific to IL-6 have been shown to be present on all of these cell types (15), and a cDNA for a human IL-6 receptor has been isolated and sequenced (16). Although extensive research has been devoted to studying the regulation of IL-6 expression, tissue distribution of IL-6, and effects of IL-6 on its various target cell types, no x-ray structure has been determined, and reports relating structural features of the IL-6 molecule to its biologic activity are relatively rare.

It has been reported that the post-translational modifications of IL-6 secreted by human cells include phosphorylation, sialylation, and *O*- and *N*-glycosylation (17). However, human IL-6 retains all of its major biologic activities when expressed in *E. coli*, which lacks eukaryotic post-translational mechanisms, implying that these post-translational modifications are not required for biologically active IL-6.

Sequence comparisons suggest that the four cysteines in human IL-6 may be functionally important. These cysteines represent four of the 16 amino acids that are conserved in other proteins known to have homology with human IL-6 (murine and rat IL-6, murine and human G-CSF,[3] and chicken myelomonocytic growth factor). Similar disulfide structures have been determined for human IL-6, murine IL-6, and human G-CSF (18, 19), and intact disulfide bonds have been shown to be necessary for the biologic activity of human G-CSF (20). However, we have shown previously that a rIL-6 analogue in which cys-

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[3] Abbreviations used in this paper: G-CSF, granulocyte colony stimulating factor; pen/strep, 100 IU/ml penicillin G and 100 μg/ml streptomycin; TN, 30 mM Tris (pH 7.4), 30 mM NaCl.

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teines have been replaced by serines retains the ability to induce differentiation of a mouse B cell line and stimulate fibrinogen secretion from a rat hepatoma cell line (21).

By constructing a series of N-terminal deletions in rIL-6, it has been demonstrated that deletion of amino acids 1 to 28 does not significantly reduce biologic activity, whereas deletion of amino acids 1 to 34 results in loss of biologic activity (22). These findings led to the suggestion that biologic activity might be localized to a region extending from amino acid 28 toward the C-terminus of IL-6, although the size of this region could not be determined from the available data.

In this article, we examine structure/activity relationships of human IL-6 by comparing a cysteine-free rIL-6 and a series of deletion mutants to a full-length, cysteine-containing rIL-6 in a mouse hybridoma proliferation assay, human and rat hepatocyte stimulation assays, and a human B cell differentiation assay.

MATERIALS AND METHODS

Construction of Cysteine-Free and Cysteine-Containing IL-6 Genes

The construction of the gene encoding a cysteine-free IL-6 analogue (rIL-6S) has been described previously (21). Briefly, this gene was assembled from 22 synthetic oligonucleotides and initially cloned into a modified pBS M13+ cloning vector (Stratagene, La Jolla, CA). The stop codon of the natural gene was converted to a serine codon by cassette mutagenesis with synthetic oligonucleotides to produce the plasmid p365. The IL-6 gene in this vector was subcloned into the expression vector p340 as described previously (21). This vector was constructed by replacement of the λ P_R promoter of the expression vector pJG200 (23) with the P-*trc* promoter of pKK233-2 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). A synthetic minicistron, which has been reported to increase levels of protein expression in *E. coli* (24), was inserted into the *NcoI* site immediately downstream of the P-*trc* promoter. The sequence of this minicistron, beginning with the initiating methionine and extending to the initiating methionine of the rIL-6 gene, is 5'-ATGTATCGATTAAATAAGGAGGAATAACCATG-3'. The gene encoding the cysteine-containing IL-6 analogue (rIL-6) was constructed by replacement of the *EcoRV* to *StuI* fragment of the rIL-6S gene in p365 with a fragment composed of six synthetic oligonucleotides. This fragment was identical in sequence to the original *EcoRV* to *StuI* fragment, except that the codons corresponding to the four cysteine codons of natural IL-6 were changed from serine codons to cysteine codons. After sequence verification, the rIL-6 gene from the resulting plasmid, p463, was moved into the p340 expression vector to create the plasmid p478. DNA was sequenced by the chain-termination method (25) with a commercially available kit (Sequenase Kit; United States Biochemical Corp., Cleveland, OH). All DNA manipulations were performed according to standard procedures (26).

Construction of Deletion Mutants

Deletion mutants were constructed using a previously published method for oligonucleotide-directed mutagenesis (27). This protocol was modified by use of Sequenase (United States Biochemical Corp.) in place of T4 DNA polymerase, as described previously (28). Potential mutants were screened by amplifying regions targeted for deletion with the polymerase chain reaction (29). Colonies positive for the desired deletion were grown, double-stranded DNA was prepared, and the region surrounding the deletion was sequenced. After verification of the presence of the desired deletion, the entire rIL-6 coding region was sequenced to exclude the possibility of second site mutations. Mutant genes were subcloned into the p340 expression vector using the same protocol as for rIL-6S.

Expression, Purification, and Quantitation of rIL-6 Variants

E. coli JM101 cells were transformed with expression vectors containing IL-6 variants, and single, ampicillin-resistant colonies were used to inoculate 10 ml of terrific broth (30) plus ampicillin (100 μg/ml). Cultures were grown in a shaking incubator at 37°C. When the OD_{595} reached approximately 1.5, expression of fusion proteins was induced by addition of 100 μl of isopropyl-β-D-thiogalactopyranoside (25 mg/ml), and cultures were returned to the shaking incubator. β-Galactosidase activity was measured according to a previously described protocol (31). When β-galactosidase activity leveled off, cells were harvested by centrifugation, and pellets were stored at −20°C until used for purification of fusion proteins.

Each frozen pellet was thawed, resuspended in 1 ml of TN buffer, and transferred to a 1.5-ml microcentrifuge tube. The cells were pelleted by spinning for 1 min in a microcentrifuge, and the cell pellet was resuspended in 1 ml of TN buffer. After addition of 5 μl of 0.5 M EDTA (pH 8.0) and 50 μl of lysozyme (1 mg/ml in TN), the resuspended cells were incubated for 20 min on ice. Cells were lysed by freezing and thawing twice, and 10 μl of 100 mM PMSF was added. The cell lysate was sonicated for 30 s to reduce viscosity, and the insoluble fraction of the cell lysate was pelleted by spinning in a microcentrifuge for 15 min.

The pellet was resuspended by sonication in 500 μl of PBS without calcium and magnesium. The fusion protein was solubilized by adding 500 μl of 4% sodium lauroylsarcosine in PBS, and insoluble contaminants were removed by spinning in a microcentrifuge for 1 min. The fusion protein was precipitated by addition of 500 μl of a saturated solution of ammonium sulfate and incubation on ice for 30 min. The precipitated protein was pelleted by spinning in a microcentrifuge for 15 min. The resulting pellet was resuspended and reprecipitated in the same manner as the initial insoluble lysate pellet, except that the sodium lauroyl sarcosine concentration was 0.3% rather than 4%. The final pellet was resuspended in 100 μl of PBS, transferred to a siliconized microcentrifuge tube, and stored at 4°C until used for bioassay or quantitation.

Before bioassay or quantitation, the IL-6 variants were cleaved from β-galactosidase by incubating 20 μl of the protein preparation with 6 μl of collagenase (from *Achromobacter iophagus*; 1 mg/ml in 10 mm Tris (pH 7.4), 250 mm NaCl, 1 mm $CaCl_2$; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 45 min at room temperature. Immediately after collagenase treatment, the proteins were either diluted in the appropriate tissue culture medium and assayed for biologic activity or prepared for quantitation. Collagenase alone, at the concentration used to cleave the fusion protein, did not have a detectable effect in the bioassays.

Proteins were quantitated by SDS/PAGE under reducing conditions followed by Coomassie staining and scanning laser densitometry (UltroScan XL; LKB, Bromma, Sweden). Peak areas were converted to protein concentrations using a BSA standard curve. Typical protein yields were 10 to 20 μg/10-ml culture.

IL-6 Bioassays

Hybridoma growth assay. The hybridoma growth factor activity was measured using an IL-6-dependent, mouse-mouse hybrid cell line (7TD1) as described previously (32). Briefly, 7TD1 cells were suspended at a density of 2×10^4 cells/ml in Iscove's medium (GIBCO) containing 10% FCS, L-arginine (0.55 mM), L-asparagine (0.24 mM), L-glutamine (1.5 mM), 2-ME (50 μM), hypoxanthine (0.1 mM), thymidine (16 μM), and pen/strep. Cells were plated 100 μl/well in 96-well tissue culture plates. IL-6 samples to be tested were diluted in the same medium, and each dilution was tested in duplicate by adding 100 μl/well to the plated cells. After 4 or 5 days, the number of cells was evaluated by colorimetric determination of hexosaminidase levels (33). Plates were read at a wavelength of 405 nm on a V_{max} ELISA plate reader (Molecular Devices Corp., Palo Alto, CA).

B cell differentiation assay. The human EBV transformed B cell line, SKW6.4 (34), was maintained in RPMI medium 1640 (GIBCO) supplemented with 10% FCS and pen/strep. For determination of B cell differentiation activity, cells were diluted to a density of 6×10^4 cells/ml in maintenance medium and plated 100 μl/well in 96-well tissue culture plates. IL-6 samples to be tested were diluted in the same medium, and each dilution was tested in duplicate by adding 100 μl/well to the plated cells. After 3 days conditioned medium was collected from individual wells and assayed for IgM production using a sandwich ELISA as described previously (35), except that TMB peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD) was used for color development, and plates were read at a wavelength of 450 nm on the V_{max} plate reader.

Hepatocyte stimulation assays. Hepatocyte stimulation activity was measured in rat and human hepatoma cell lines. FAZA 967 (36) rat hepatoma cells were maintained in DMEM/F12 (GIBCO) medium supplemented with 10% NuSerum (Collaborative Research, Lexington, MA) and pen/strep. On the day before treatment, cells were trypsinized and plated on 96-well tissue culture plates at a density sufficient to give confluent monolayers after overnight growth. The next day, medium was removed and replaced with 100 μl/well of serum-free DMEM/F12 supplemented with pen/strep. IL-6 samples to be tested were diluted in the same medium supplemented with

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10^{-6} M dexamethasone, and each dilution was tested in duplicate by adding 100 μl/well to the plated cells. Conditioned medium was collected after 24 h and assayed for fibrinogen production using a sandwich ELISA. A commercial polyclonal antibody (goat anti-rat fibrinogen; Cappel, Durham, NC) was used for fibrinogen capture, and detection of bound fibrinogen was accomplished in two steps with rabbit anti-rat fibrinogen polyclonal antiserum (obtained from Gerald R. Crabtree, Stanford University) followed by affinity-purified horseradish peroxidase conjugated goat anti-rabbit IgG (Cappel). Color was developed and plates were read as in the B cell differentiation assay.

HEP 3B2 human hepatoma cells (37) were maintained in DMEM-H medium supplemented with 10% FCS and pen/strep. Cells were plated identically to FAZA 967 cells, except that the medium was not replaced before treatment, and IL-6 samples to be tested were diluted in maintenance medium supplemented with 10^{-6} M dexamethasone. Conditioned medium was collected after 48 h and assayed for fibrinogen production by a sandwich ELISA using commercially available capture antibody (IgG fraction, goat anti-human fibrinogen; Cappel) and peroxidase-conjugated detection antibody (peroxidase-conjugated IgG fraction, goat anti-human fibrinogen; Cappel). Color was developed and plates were read as in the B cell differentiation assay.

Calculation and Comparison of Biologic Activities

All assays were carried out on duplicate serial dilutions of two or three independent preparations of each IL-6 variant. The biologic activities were determined from dose response curves prepared for each protein preparation. The activity of each IL-6 variant was calculated by taking the mean of the activities determined for independent protein preparations, and the mean activities of different mutants were compared using a two-sample *t* test.

For the 7TD1 assay, OD_{405} was assumed to be directly proportional to cell number and was plotted directly against the log of the IL-6 concentration. For the IgM and fibrinogen ELISA assays, OD_{450} was converted to protein concentration using the V_{max} plate reader software before being plotted. For each of the four assays, the roughly linear portion of each dose-response curve was fitted with a second order polynomial curve using a computer program. The IL-6 concentration that gave half maximal proliferation in the 7TD1 assay was calculated for each mutant, and this value was used to calculate hybridoma growth activity relative to the full length cysteine-containing IL-6 analogue. For the B cell differentiation and hepatocyte stimulation assays, the IL-6 concentration that caused a doubling of IgM or fibrinogen secretion was calculated, since this value allowed a more accurate comparison of biologic activities, especially for relatively inactive mutants.

RESULTS

Construction of rIL-6 mutants. In order to examine the structural requirements for biologically active IL-6, we have constructed genes encoding three types of rIL-6 mutants: 1) a mutant referred to as rIL-6S, in which the four naturally occurring cysteines are replaced by serines; 2) a series of mutants, each containing an internal deletion of 20 amino acids; and 3) a cysteine-free mutant with amino acids 4 to 23 deleted. The deletion mutants are named according to the first and last amino acids of the deletion (e.g., rIL-$6_{\Delta 4\text{-}23}$). The mutants of the second type constitute a set of nine non-overlapping deletions that span the length of the IL-6 gene. We have also constructed a gene encoding nondeleted, cysteine-containing rIL-6 for use as a standard.

The construction of rIL-6S has been described previously (21). The rIL-6S gene was assembled from 22 synthetic oligonucleotides and has a sequence based on that of the cDNA for human IL-6. However, several point mutations have been introduced to create new restriction sites without changing the encoded amino acid sequence. These new restriction sites have been incorporated to facilitate assembly of the rIL-6S gene and to allow mutagenesis by replacement of entire sections of the gene. In the gene encoding cysteine-containing rIL-6, the *Eco*RV-*Stu*I fragment of the rIL-6S gene has been replaced with an analogous synthetic fragment encoding the natural pattern of cysteines. To enhance expression of the encoded rIL-6 in *E. coli*, several changes have been made in the amino acid sequence of the encoded rIL-6 with respect to natural, human IL-6: the natural signal peptide has been replaced by an initiating methionine, circumventing the need for post-translational processing normally performed by eukaryotic cells; and the natural termination codon has been converted to a serine codon to allow expression of rIL-6 as a fusion protein.

Deletions have been introduced into the rIL-6 and rIL-6S genes by oligonucleotide-directed mutagenesis. Deletion mutants are shown diagrammatically in Figure 1. Nine of these mutants were constructed by deleting segments of the cysteine-containing rIL-6 gene. These deletions were chosen, in the absence of detailed structural data, to allow a systematic determination of which regions of the IL-6 protein are required for activity. Deletion of 20 amino acids at a time allowed examination of the entire length of the IL-6 molecule by constuction of a practical number of mutants, and both deletions that include cysteines (rIL-$6_{\Delta 44\text{-}63}$ and rIL-$6_{\Delta 64\text{-}83}$) remove both members of one disulfide bond, thus avoiding possible

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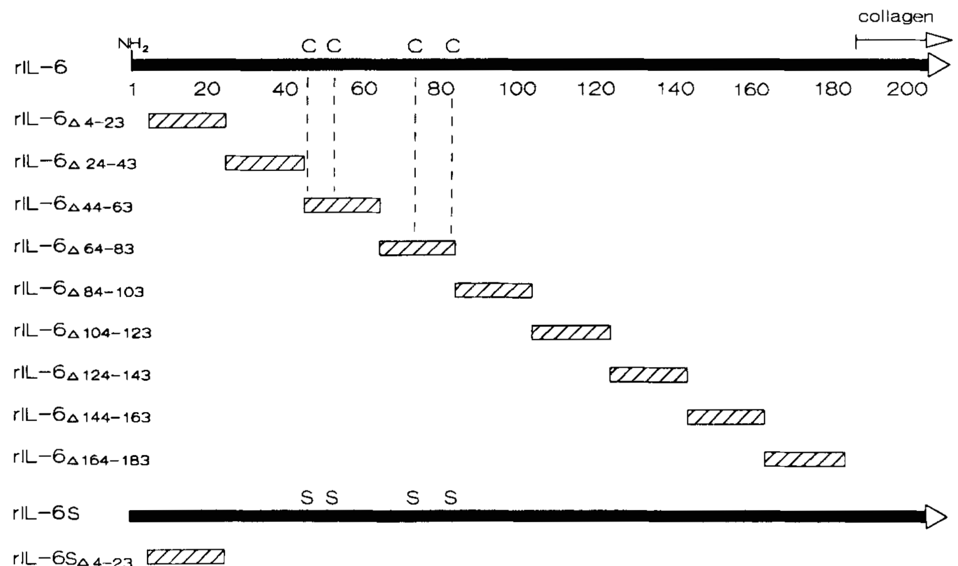

Figure 1. Schematic representation of rIL-6 variants. rIL-6 is the nonmutated protein used for determination of the relative biologic activities of the mutants. *Hatched boxes* represent deleted segments in the mutant proteins. *Subscripted numbers* in the names of mutants refer to the first and last amino acids encompassed by deletions. Numbers are based on the positions of analogous amino acids in natural human IL-6 and do not include the initiating methionine, which replaces the naturally occurring signal peptide. Therefore, the first deletion actually begins with the fifth amino acid encoded by the rIL-6 gene. We have shown previously, however, that the initiating methionine is removed by *E. coli* from >50% of the expressed fusion protein (22). C represents the position of cysteine residues in rIL-6, and *vertical dashed lines* indicate which deletions include cysteines. S represents the serines that replace the naturally occurring cysteines in rIL-6S and rIL-$6S_{\Delta 4\text{-}23}$.

problems associated with unpaired cysteines. The activity of one cysteine-free deletion mutant, rIL-$6S_{\Delta 4\text{-}23}$, was also examined. Expression of each of the rIL-6 variants was achieved by subcloning into a vector that directs the synthesis of a tripartite protein composed of rIL-6, a 60-amino acid collagen linker, and β-galactosidase.

Purification and determination of rIL-6 concentration. To facilitate testing of large numbers of rIL-6 mutants, we developed a protocol for partial purification of rIL-6 from small cultures of *E. coli* transformed with rIL-6 genes in the p340 expression vector. This protocol consists essentially of detergent solubilization of the insoluble fraction of the cell lysate followed by selective ammonium sulfate precipitation of the fusion protein. The rIL-6 is released from the fusion protein by treatment with collagenase. This protocol yields approximately 10 to 20 μg of rIL-6 from one 10-ml culture of *E. coli*. The resulting IL-6 can be used in bioassays at concentrations of up to 50 μM without noticeable toxic or stimulatory effects of contaminating *E. coli* components. Using rIL-6 prepared in this way, we are able to measure reliably the activity of mutant proteins with activity as low as 0.001% that of the non-mutated rIL-6.

The large yields of rIL-6 obtained with this procedure permit quantitation by densitometry of Coomassie stained gels. This technique has several advantages over Western blotting, which has been used when protein concentrations are too low to visualize proteins by direct staining: 1) by comparison with an albumin standard curve, we can estimate molar concentrations as well as relative concentrations of the rIL-6 variants; and 2) Western blotting relies on antibody recognition, which may be altered by mutations in unpredictable ways.

Measurement of biologic activity. To determine the effects of specific mutations on biologic activity, and whether these effects are tissue- or species-specific, we tested the rIL-6 variants in four representative bioassays: a mouse hybridoma proliferation assay (7TD1), a human B cell differentiation assay (SKW6.4), a rat hepatocyte stimulation assay (FAZA), and a human hepatocyte stimulation assay (HEP 3B2). For each variant, the assays were carried out in duplicate on two or three protein samples prepared independently from separate bacterial cultures.

To compare the effects of different mutations on the activity of rIL-6, we have calculated sp. act. for the mutants in each of the four bioassays. For the mouse hybridoma assay we have defined sp. act. based on the concentration of rIL-6 required to induce half-maximal stimulation of proliferation. For the human B cell differentiation assay and the human and rat hepatocyte stimulation assays, we have defined sp. act. based on the amount of rIL-6 required to double the secretion of IgM or fibrinogen, respectively. For this latter group of assays, this means of comparison is preferable to that based on half maximal stimulation for several reasons. In the B cell and hepatocyte assays, a clear maximum was not achieved with the nonmutated rIL-6, even at the highest protein concentration, making accurate determination of maximal stimulation difficult. For the less active variants, the level of stimulation achieved with the highest concentrations tested was often less than half of the maximal stimulation achieved with rIL-6, making it impossible to compare activities based on half maximal stimulation. The concentration of rIL-6 that doubles secretion in the B cell and hepatocyte assays is approximately equal to the lowest detectable concentration, whereas the concentration that gives half maximal stimulation may be several orders of magnitude higher. Therefore, the concentration of rIL-6 that doubles secretion gives a reasonable estimate of the sensitivity of these assays. The concentration of rIL-6 that gives half maximal proliferation in the hybridoma growth assay provides a comparable estimate of sensitivity, since the concentration that gives maximal stimulation is only slightly higher than the lowest concentration that gives a detectable effect.

We have defined mutants that give half maximal stimulation in the 7TD1 assay or that stimulate a doubling of protein secretion in the hepatocyte and B cell assays to be active in these assays. The sp. act. of mutants that were active in all four of the bioassays are shown graphically in Figure 2.

Comparison of biologic activities of rIL-6 variants.

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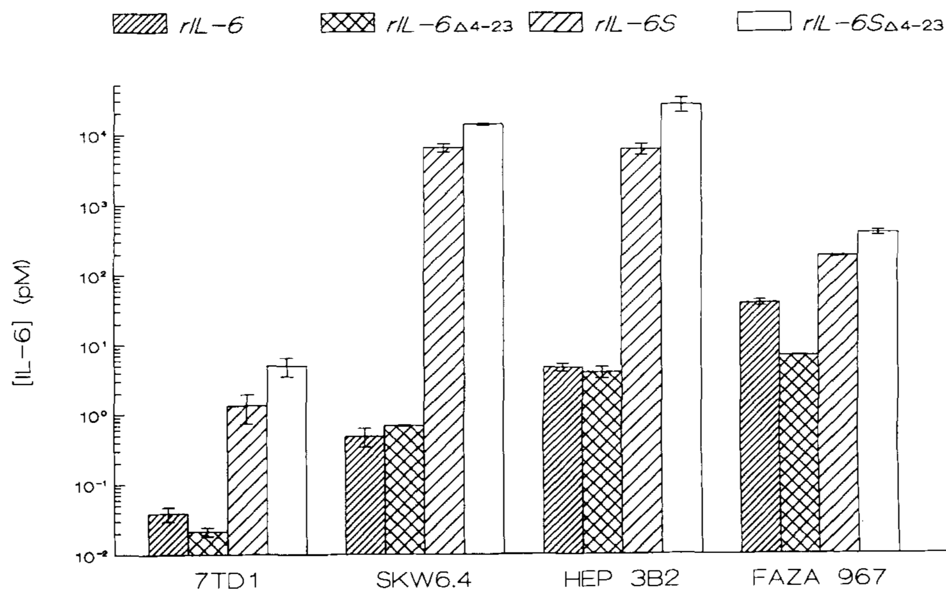

Figure 2. Sp. act. of biologically active variants. Activity in the 7TD1 assay is defined as the concentration of rIL-6 needed to cause half maximal proliferation. Activity in the HEP 3B2 and FAZA 967 assays is defined as the concentration of rIL-6 needed to cause a doubling of secreted fibrinogen. Note that the vertical axis represents *[IL-6]* on a logarithmic scale. Activity in the SKW6.4 assay is defined as the concentration of rIL-6 needed to cause a doubling of secreted IgM. Activities were calculated as described in *Materials and Methods* and *error bars* represent the SEM.

TABLE I
Biologic activities of rIL-6 mutants as percent activity of rIL-6

Mutant[a]	7TD1[b]	FAZA 967[c]	HEP 3B2[c]	SKW6.4[d]
rIL-6	100	100	100	100
rIL-$6_{\Delta 4-23}$	320	560	117	68
rIL-$6_{\Delta 24-43}$	0.0013	<0.2	<0.02	<0.002
rIL-$6_{\Delta 44-63}$	<0.001	<1	<0.1	<0.01
rIL-$6_{\Delta 64-83}$	<0.0001	<0.1	<0.01	<0.001
rIL-$6_{\Delta 84-103}$	<0.0001	<0.1	<0.01	<0.001
rIL-$6_{\Delta 104-123}$	0.0099	<0.5	<0.06	<0.006
rIL-$6_{\Delta 124-143}$	<0.003	<3	<0.3	<0.03
rIL-$6_{\Delta 144-163}$	<0.0006	<0.6	<0.08	<0.008
rIL-$6_{\Delta 164-183}$	<0.0004	<0.4	<0.05	<0.005
rIL-6S	2.9	21	0.075	0.008
rIL-$6S_{\Delta 4-23}$	0.77	9.8	0.018	0.0035

[a] Activities for all mutants are given as a percentage of the activity of rIL-6, which has been assigned an activity of 100% in all of the bioassays. For mutants with no detectable activity, activity is given as <*c*, where *c* is a value based on the highest concentration of the mutant tested. Each deletion mutant contains a single internal deletion. Subscripted numbers in the names of mutants refer to the first and last amino acids encompassed by deletions. Positions of deleted amino acids are numbered based on the sequence of mature human IL-6. In mutants rIL-6S and rIL-$6S_{\Delta 4-23}$, the four naturally occurring cysteines have been converted to serines.

[b] Activity in the 7TD1 assay is calculated as described in *Materials and Methods*, based on the concentration of rIL-6 needed to cause half maximal proliferation.

[c] Activity in the HEP 3B2 assay and the FAZA 967 assay is calculated as described in *Materials and Methods*, based on the concentration of rIL-6 needed to cause doubling of the concentration of secreted fibrinogen.

[d] Activity in the SKW6.4 assay is calculated as described in *Materials and Methods*, based on the concentration of rIL-6 needed to cause doubling of the concentration of secreted IgM.

The biologic activities of the mutant rIL-6 proteins are shown in Table I as a percentage of the activity of non-mutated rIL-6, which has been set at 100% for each assay. The relative activity of rIL-6S was reduced in all four of the biologic assays, although this reduction was much more significant in the human cell lines than in the rodent cell lines. Of the deletion mutants only rIL-$6_{\Delta 4-23}$ retained measurable activity in all of the bioassays. Although the activity of this mutant was similar to that of rIL-6 in the human hepatocyte assay ($p > 0.4$) and human B cell assay ($p > 0.2$), its activity was increased in the mouse hybridoma assay ($p < 0.05$) and the rat hepatocyte assay ($p < 0.005$). The same deletion was introduced into the gene encoding cysteine-free rIL-6S to create the mutant rIL-$6S_{\Delta 4-23}$. Although the deletion of amino acids 4 to 23 did not reduce the activity of the cysteine-containing protein, the activity of rIL-$6S_{\Delta 4-23}$ was reduced significantly in comparison with that of rIL-6S when assayed on 7TD1 ($p < 0.05$), FAZA 967 ($p < 0.01$), HEP 3B2 ($p < 0.025$), and SWK6.4 ($p < 0.005$) cells. Of the other deletion mutants, only rIL-$6_{\Delta 24-43}$ and rIL-$6_{\Delta 104-123}$ retained measurable activity. This activity was only detected in the murine hybridoma assay, presumably because of the high sensitivity of this assay, and in both cases activity was reduced to <0.01%.

DISCUSSION

IL-6 has been demonstrated to exert a number of effects on a variety of tissue types. We are interested in determining whether these biologic activities can be correlated with structural features of the IL-6 protein and, in particular, whether structural requirements for activity might vary between tissues or between animal species. We therefore examined the effects of mutations on representative biologic activities of a human rIL-6 analogue. The mutations introduced into rIL-6 are of three types: 1) replacement of cysteines by serines, 2) deletion of 20 amino acid segments throughout the length of the cysteine-containing rIL-6, and 3) a cysteine-free mutant containing a 20 amino acid deletion at the N-terminus.

All of these mutants were expressed as β-galactosidase fusion proteins in *E. coli*. The relatively high yields of rIL-6 obtainable with this system allowed direct quantitation of rIL-6 concentrations by dye binding and detection of activities as low as 0.001% of non-mutated rIL-6. The simplicity of the purification protocol, which requires no chromatography or reduction and reformation of disulfide bonds, allowed preparation and testing of multiple samples of each variant. Each protein preparation was tested in four bioassays: a mouse hybridoma growth assay (7TD1), a human B cell differentiation assay (SKW6.4), a human hepatocyte stimulation assay (HEP 3B2), and a rat hepatocyte stimulation assay (FAZA 967).

The mutant in which cysteines were replaced by serines (rIL-6S) allowed us to address the role of disulfide bonds in maintaining the active form of IL-6. We reported previously that rIL-6S retains activity in a murine B cell differentiation assay and a rat hepatocyte stimulation assay. In this report we compare the activity of rIL-6S with the cysteine-containing analogue, rIL-6, and show that removal of cysteines results in a significant loss of activity in all four of the bioassays. The four cysteines in human IL-6 are conserved in murine and rat IL-6 and in the other proteins known to have significant homology with IL-6 (human and murine G-CSF and chicken monomyelocytic growth factor). It has also been demonstrated that the disulfide structures of human IL-6, murine IL-6, and human G-CSF are similar (18–20) and that substitution of any one of the four conserved cysteines in human G-CSF results in loss of activity (20). It is, therefore, not surprising that the preclusion of disulfide bond formation in rIL-6S results in decreased activity. What is unexpected, however, is that the removal of cysteines resulted in a significantly greater loss of activity for the human than for the rodent cell lines. Relative to rIL-6, rIL-6S retained approximately 20% activity in the rat hepatocyte assay and 3% activity in the mouse hybridoma assay, whereas its relative activity was reduced to less than 0.1% in the human hepatocyte assay and less than 0.01% in the human B cell assay. The finding that murine IL-6 is not active in human cells (38) indicates that human, but not rodent, cells require some structural feature unique to human IL-6. It is possible that this feature is disrupted by removal of cysteines, thereby decreasing activity in human but not rodent cells. It is also possible that rIL-6S is more sensitive to degradation by the rodent cell lines.

To determine which regions of the IL-6 molecule are required for biologic activity, we constructed nine deletion variants of cysteine-containing rIL-6. Each of these variants contains one of a series of 20 amino acid deletions that, taken as a group, span the length of the rIL-6 gene. Of the deletion mutants tested, only rIL-$6_{\Delta 4-23}$ retained significant activity. This is consistent with previous findings, which showed that an IL-6 mutant containing an N-terminal deletion of 28 N-terminal amino acids retains biologic activity (22). However, these findings left unanswered the question of what fraction of the remaining sequence is necessary for activity. Each of the eight deletions we made in this region abolished activity in all of four of our bioassays, strongly suggesting that

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this entire region is necessary for all of the activities that we have measured. Interestingly, although rIL-$6_{\Delta 4\text{-}23}$ retained activity similar to rIL-6 in the human assays, its activity was increased relative to rIL-6 in both of the rodent assays. This may be related to the fact that the sequence of human IL-6 shows the highest degree of divergence from the sequences of mouse and rat IL-6 at the amino terminus. It is possible that removal of part of this divergent sequence from human IL-6 raises its affinity for murine cells to a value similar to that of murine IL-6, which has been demonstrated to have fivefold higher affinity for murine cells (38).

To see whether the effects of the mutations in rIL-6S and rIL-$6_{\Delta 4\text{-}23}$ are independent, we tested a cysteine-free mutant in which amino acids 4 to 23 had been deleted (rIL-$6S_{\Delta 4\text{-}23}$). If these two types of mutations exert their effects independently, then, in the rodent cell lines, the increased activity caused by deletion of amino acids 4 to 23 might be expected to compensate for the loss of activity caused by the absence of cysteines. However, we found rIL-$6S_{\Delta 4\text{-}23}$ to be significantly less active than rIL-$6_{\Delta 4\text{-}23}$ in all four of the bioassays. This suggests that, although amino acids 4 to 23 do not contribute to the biologic activity of cysteine-containing rIL-6, they may play some role in stabilizing the biologically active conformation of the molecule in the absence of disulfide bonds.

Our results provide evidence that disulfide bonding is important in maintaining the biologically active conformation of human IL-6, although removal of cysteines causes a significantly greater loss of activity on human cells than on rodent cells. Our results also indicate that, with the exception of the short segment at the amino terminus, which has been shown previously to be unnecessary for biologic activity, the entire primary sequence of IL-6 is required for activity. Finally, our findings indicate the existence of species specificity but not tissue specificity in the structural requirements for biologically active human IL-6. More refined studies with point mutants will be required to determine precisely which parts of the primary amino acid sequence are most important for activity. Toward this end we have generated a library of rIL-6 genes containing random point mutations, and members of this library are currently being sequenced and examined for biologic activity. Binding studies will also be necessary to determine whether the effects of mutations on biologic activity are the result of changes in affinity for or activation of IL-6 receptors. The ability to separate binding from activation might allow the design of IL-6 antagonists. Such antagonists would facilitate studies of IL-6 function in intact animals and might also prove clinically useful in suppression of specific immune reactions.

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REFERENCES

1. **Ritchie, D. G., and G. M. Fuller.** 1983. Hepatocyte-stimulating factor: a monocyte-derived acute phase regulatory protein. *Ann. NY Acad. Sci. 408:490.*
2. **Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamuba, N. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto.** 1986. Complementary DNA for a novel interleukin that induces B lymphocytes to produce immunoglobulin. *Nature 324:73.*
3. **Weissenbach, J., Y. Chernajovsky, M. Zeevi, L. Shuman, Y. Soreq, U. Nir, D. Wallach, M. Perricaudet, P. Tiollais, and M. Revel.** 1980. Two interferon mRNAs in human fibroblasts: in vitro translation and *Escherichia coli* cloning studies. *Proc. Natl. Acad. Sci. USA 77:7152.*
4. **Kupper, T. S., K. Min, P. Sehgal, H. Mizutani, N. Birchall, A. Ray, and L. May.** 1989. Production of IL-6 by keratinocytes: implications for epidermal inflammation and immunity. *Ann. NY Acad. Sci. 557:454.*
5. **Podor, T. J., F. R. Jirik, D. L. Loskutoff, D. A. Carson, and M. Lotz.** 1989. Human endothelial cells produce IL-6: lack of responses to exogenous IL-6. *Ann. NY Acad. Sci. 557:374.*
6. **Poupart, P., P. Vandenabeele, S. Cayphas, J. Van Snick, G. Haegeman, V. Kruys, W. Fiers, and J. Content.** 1987. B cell growth modulating and differentiating activity of recombinant human 26-kd protein (BSF-2, HuIFN-β_2, HPGF). *EMBO J. 6:1219.*
7. **Geiger, T., T. Andus, J. Bauer, H. Northogg, U. Ganter, T. Hirano, T. Kishimoto, and P. C. Heinrich.** 1988. Cell-free-synthesized interleukin-6 (BSF-2/IFN-β_2) exhibits hepatocyte-stimulating activity. *Eur. J. Biochem. 175:181.*
8. **Matsuura, Y., M. Tatsumi, K. Enami, S. Morikawa, S. Yamazaki, and M. Masayoshi.** 1989. Expression of IL-6/IFN-β_2 in a Baculovirus system and its biological function. *Ann. NY Acad. Sci. 557:122.*
9. **Brakenhoff, J. P. J., E. R. De Groot, R. F. Evers, H. Pannekoek, and L. A. Aarden.** 1987. Molecular cloning and expression of hybridoma growth factor in *E. coli. J. Immunol. 139:4116.*
10. **Hirano, T., T. Taga, N. Nakano, K. Yasukawa, S. Kashiwamura, K. Shimizu, K Nakajima, K. H. Pyun, and T. Kishimoto.** 1985. Purification to homogeneity and characterization of human B cell differentiation factor (BCDF or BSF-2). *Proc. Natl. Acad. Sci USA 82:5490.*
11. **Hodgkin, P., M. Bond, A. O'Garra, G. Frank, F. Lee, R. Coffman, A. Zlotnik, and M. Howard.** 1988. Identification of IL-6 as a T cell-derived factor that enhances the proliferative response of thymocytes to IL-4, and phorbol myristate acetate. *J. Immunol. 141:151.*
12. **Okada, M., M. Kitahara, S. Kishimoto, T. Matsuda, T. Hirano, and T. Kishimoto.** 1988. IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. *J. Immunol. 141:1543.*
13. **Van Damme, J., G. Opdenakker, R. J. Simpson, M. R. Rubira, S. Cayphas, A. Vink, A. Billiau, and J. Van Snick.** 1987. Identification of the human 26-kD protein, interferon β_2 (IFNβ_2), as a B-cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. *J. Exp. Med. 165:919.*
14. **Ikebuchi, K., G. G. Wong, S. C. Clark, J. N. Ihle, Y. Hirai, and M. Ogawa.** 1987. Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proc. Natl. Acad. Sci. USA 84:9035.*
15. **Taga, T., K. Kawanishi, R. R. Hardy, T. Hirano, and T. Kishimoto.** 1987. Receptors for B cell stimulatory factor 2 (BSF-2): quantitation, specificity, distribution and regulation of the expression. *J. Exp. Med. 166:967.*
16. **Yamasaki, K., T. Taga, Y. Hirata, H. Yawata, Y. Kawanishi, B. Seed, T. Taniguchi, T. Hirano, and T. Kishimoto.** 1988. Cloning and expression of the human interleukin-6 (BSF-2/IFNβ_2) receptor. *Science 241:825.*
17. **Santhanam, U., J. Ghrayeb, P. B. Sehgal, and L. T. May.** 1989. Post-translational modifications of human interleukin-6. *Arch. Biochem. Biophys. 274:161.*
18. **Simpson, R. J., and R. L. Moritz.** 1988. Characterization of a recombinant murine interleukin-6: assignment of disulfide bonds. *Biochem. Biophys. Res. Commun. 157:364.*
19. **Clogston, L. C., T. C. Boone, C. Crandall, E. A. Mendiaz, and H. S. Lu.** 1989. Disulfide structures of human interleukin-6 are similar to those of human granulocyte colony stimulation factor. *Arch. Biochem. Biophys. 272:144.*
20. **Lu, H. S., T. C. Boone, L. M. Souza, and P. H. Lai.** 1989. Disulfide and secondary structures of recombinant human granulocyte colony stimulating factor. *Arch. Biochem. Biophys. 268:81.*
21. **Jambou, R. C., J. N. Snouwaert, G. A. Bishop, J. R. Stebbins, J. A. Frelinger, and D. M. Fowlkes.** 1988. High-level expression of a bioengineered, cysteine-free hepatocyte-stimulating factor (interleukin 6)-like protein. *Proc. Natl. Acad. Sci. USA 85:9426.*
22. **Brakenhoff, J. P. J., M. Hart, and L. A. Aarden.** 1989. Analysis of human IL-6 mutants expressed in *Escherichia coli*: biologic activities are not affected by deletion of amino acids 1–28. *J. Immunol. 143:1175.*
23. **Germino, J., and D. Bastia.** 1984. Rapid purification of a cloned gene product by genetic fusion and site-specific proteolysis. *Proc. Natl. Acad. Sci. USA 81:4692.*
24. **Schoner, B. E., R. M. Belagaje, and R. G. Schoner.** 1986. Translation of a synthetic two-cistron mRNA in *Escherichia coli. Proc. Natl. Acad. Sci. USA 83:8506.*
25. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:5463.*

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26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol. 154:367.*
28. **Schena, M.** 1988. High efficiency oligonucleotide-directed mutagenesis using Sequenase. *U.S. Biochem. Corp. Editorial Comments. 15:23.*
29. **Zon, L. I., D. M. Dorfman, and S. H. Orkin.** 1989. The polymerase chain reaction colony miniprep. *Biotechniques 7:695.*
30. **Tartof, K. D., and C. A. Hobbs.** 1987. Improved media for growing plasmid and cosmid clones. *Bethesda Res. Lab. Focus 9:12.*
31. **Miller, J. H., ed.** 1972. *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 352.
32. **Van Snick, J., S. Cayphas, A. Vink, C. Uyttenhove, P. G. Coulie, M. R. Rubira, and R. J. Simpson.** 1986. Purification and NH_2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. *Proc. Natl. Acad. Sci. USA 83:9679.*
33. **Landegren, U.** 1984. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: applications to detection of lymphokines and cell surface antigens. *J. Immunol. Methods. 67:379.*
34. **Saiki, O., and P. Ralph.** 1983. Clonal differences in response to T cell replacing factor (TRF) for IgM secretion and TRF receptors in a human B lymphoblast cell line. *Eur. J. Immunol. 13:31.*
35. **Kanowith-Klein, S., A. Saxon, and C. H. Uittenbogaart.** 1987. Constitutive production of B cell differentiation factor-like activity by human T and B cell lines. *Eur. J. Immunol. 17:593.*
36. **Deschartrette, J., and M. C. Weiss.** 1974. Characterization of differentiated and dedifferentiated clones from a rat hepatoma. *Biochimie 56:1603.*
37. **Darlington, G. J., D. R. Wilson, and L. B. Lachman.** 1986. Monocyte conditioned medium, interluekin-1, and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. *J. Cell Biol. 103:787.*
38. **Coulie, P. G., M. Stevens, and J. V. Snick.** 1989. High- and low-affinity receptors for murine interleukin 6: distinct distribution on B and T cells. *Eur. J. Immunol. 19:2107.*

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