Immunochemical Mapping of Domains in Human Interleukin 4 Recognized by Neutralizing Monoclonal Antibodies

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ABSTRACT: Human interleukin 4 is a highly pleiotropic cytokine secreted by activated T cells that exerts multiple biological effects on B and T lymphocytes and other cell types. Elucidation of structure–function relations was accomplished by epitope mapping of a panel of monoclonal antibodies and by mutagenesis of selected amino acid residues. Epitope mapping of these monoclonal antibodies was achieved through binding studies with recombinant human interleukin 4 (rhuIL-4), proteolytic fragments produced by digestion with *Staphylococcus aureus* V8 protease and synthetic peptides derived from the sequence of the parent molecule. Monoclonal antibodies 25D2, 35F2, and 11B4 neutralized the in vitro T-cell proliferation activity of rhuIL-4 and also prevented binding of rhuIL4 to its cell surface receptor. These antibodies recognized sequences 104–129, 70–92, and 61–82, respectively. These regions comprise the BC loop/helix C (residues 61–92) and helix D (residues 104–129). A nonneutralizing monoclonal antibody (1A2) recognized a nonoverlapping region (residues 43–59) comprising almost entirely helix B. Mutagenesis of a cluster of residues within helix C showed that at least three residues (K84, R88, and N89) were potentially involved in receptor recognition. The existence of two distinct nonneighboring binding domains in the three-dimensional structure of rhuIL-4 provided preliminary evidence for a model of receptor interaction involving the formation of a ternary complex consisting of two molecules of the extracellular portion of the receptor and one molecule of rhuIL-4.

Human interleukin 4 (huIL-4)[1] is a cytokine primarily secreted by T lymphocytes in response to antigenic stimulation. Similar to its murine counterpart, which was originally described as a B-cell growth factor (BCGF-I) or B-cell stimulatory factor (BSF-1) (Howard et al., 1982; Paul, 1987), huIL-4 is a pleiotropic factor exhibiting a wide range of biological activities. For example, huIL-4 enhances the proliferation of activated B (Defrance et al., 1987a) and T lymphocytes (Spits et al., 1987) and induces expression of class II major histocompatibility antigens and the low-affinity receptor for IgE (CD23) on B cells (Rousset et al., 1988; Defrance et al., 1987b) and monocytes (Littman et al., 1989; te Velde et al., 1988). HuIL-4 is the principal regulator of the production of IgE and induces the production of both IgG and IgM (Pene et al., 1988). Myeloid functions are also affected by huIL-4, as evidenced by the downregulation of the production of proinflammatory cytokines (IL-1, IL-6, IL-8, and TNF) and prostaglandin E2 from monocytes (te Velde et al., 1990) and enhancement of the phagocytic function of both monocytes and granulocytes (Boey et al., 1989; Grace et al., 1989). Several other in vitro effects demonstrated the potential usefulness of huIL-4 in cancer therapy. Thus, for example, huIL-4 can inhibit the IL-2-dependent proliferation of chronic lymphocytic B cells (Karray et al., 1988), can enhance the growth of tumor-infiltrating lymphocytes specific for autologous melanoma (Kawahami et al., 1988), and can induce the activity of lymphokine-activated killer cells following preactivation with IL-2 (Kawahami et al., 1989). The expression of all biological activities appears to be mediated through binding to specific high-affinity receptors, which have recently been cloned and characterized (Izerda et al., 1990; Galizzi et al., 1990).

The availability of milligram quantities of purified rhuIL-4 (Yokota et al., 1986; Le et al., 1988) has provided a basis for structural analysis and elucidation of structure–function relations. The three-dimensional structure of *Escherichia coli*-derived rhuIL-4 has been determined by X-ray crystallography at 2.35-Å resolution (Cook et al., 1991; Walter et al., 1992; Wlodaver et al., 1992) and is consistent with the secondary and tertiary structures determined by 2D and 3D NMR techniques (Redfield et al., 1991; Smith et al., 1992; Garrett et al., 1992; Powers et al., 1992a,b). Studies with proteolytic fragments and huIL-4 synthetic peptides have provided preliminary information on domains that are potentially important for biological activities (Le et al., 1991; Postlethwaithe & Seyer, 1991). In this report we describe the epitope mapping of four monoclonal antibodies raised to rhuIL-4 and mutagenesis studies of selected amino acid residues. The neutralizing antibodies recognized two distinct nonneighboring domains in the three-dimensional structure. At least three amino acids in these domains were shown to be potentially involved in receptor recognition. Our data are consistent with a model of huIL-4–receptor interaction involving a ternary complex of two molecules of receptor and one molecule of ligand.

EXPERIMENTAL PROCEDURES

Materials. rhuIL-4 expressed in CHO cells was purified to homogeneity to a final specific activity of 2×10^7 units/mg (Le et al., 1988). [^{3}H]Thymidine and ^{125}I-labeled Bolton–Hunter reagent were obtained from Du Pont (Boston, MA).

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[1] Abbreviations: huIL-4, human interleukin 4; rhuIL-4, recombinant human interleukin 4; BSA, bovine serum albumin; PBL, peripheral blood lymphocytes; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ABTS, 2,2′-azinobis(2-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; PHA, phytohemagglutinin; TCA, trichloroacetic acid; *t*-BOC, *tert*-butyloxycarbonyl; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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Radiolabeling of rhuIL-4 to high specific radioactivity (1500 Ci/mmol) was performed by a modification of the Bolton–Hunter method (Le et al., 1991). The Daudi and U-937 cell lines used in the receptor binding assay were purchased from the American Type Culture Collection (Rockville, MD). Omnisorb cells [10% (v/v) suspension of formalinized *Streptococcus* sp. cells bearing type III Fc receptor] and horseradish peroxidase conjugated goat IgG were purchased from Calbiochem (San Diego, CA). The microtiter plates used in radioimmunoassay studies were purchased from Dynatech (Alexandria, VA). ABTS was obtained from Boehringer Mannheim (Indianapolis, IN). Dr. John Abrams of DNAX Research Institute (Palo Alto, CA) provided the hybridomas for the rat monoclonal antibodies 11B4, 25D2, and 35F2.

Monoclonal Antibodies to RhuIL-4. The rat monoclonal antibodies 11B4, 25D2, and 35F2 (Chretien et al., 1988; Abrams et al., 1989) were obtained in highly purified form by chromatography using GammaBind G (Genex, Gaithersburg, MD) and S-Sepharose (Pharmacia, Piscataway, NJ). The mouse monoclonal antibody 1A2 was produced by a hybridoma derived from the fusion of NS-1 myeloma cells and spleen cells from a Balb/c mouse immunized with rhuIL-4 (Goding, 1980). The antibody was purified from ascites fluids of Balb/c mice to higher than 95% homogeneity in a two-step procedure employing ammonium sulfate precipitation and chromatography on Affi-Gel protein A (Bio-Rad, Richmond, CA).

Synthetic Peptides and Proteolytic Fragments of RhuIL-4. All synthetic peptides representing various regions spanning the mature huIL-4 amino acid sequence were prepared by solid-phase synthesis. Synthetic huIL-4 peptides 43–59, 52–65, 61–82, 70–92, 83–104, and 97–117 were synthesized by *t*-BOC chemistry using an Applied Biosystems peptide synthesizer, model 430A (Foster City, CA). Purification was performed by reversed-phase FPLC using a PepRPC HR16/10 column (Pharmacia, Piscataway, NJ). Homogeneity of the purified peptides was confirmed by Edman degradation using an Applied Biosystems protein sequencer, model 470; by amino acid composition analysis using a Hewlett-Packard Amino Quant system; and by fast-atom-bombardment mass spectrometry. Synthetic huIL-4 peptides 1–26, 32–39, 104–112, 104–129, 113–120, and 121–126 were purchased from Bachem California (Torrance, CA). Proteolytic fragments of rhuIL-4, namely, residues 20–103, 27–103, and 44–103, were obtained by selective cleavage with *Staphylococcus aureus* V8 protease in 0.05 M ammonium bicarbonate buffer containing 0.01% SDS. Isolation and characterization of the resulting fragments were as described previously (Le et al., 1991).

DNA Synthesis and Sequencing. Synthesis of oligonucleotides was performed with an Applied Biosystems Model 380A DNA synthesizer. Purification was accomplished with the oligonucleotide purification cartridges according to specifications recommended by the manufacturer (Applied Biosystems, Foster City, CA). DNA sequencing was performed by the dideoxy method (Sanger et al., 1977) using the Sequenase kit (U.S. Biochemicals, Cleveland, OH).

ELISA. Preliminary studies of the binding of rhuIL-4 or synthetic peptides to monoclonal antibodies were performed by direct ELISA. Varying amounts of IL-4 peptides or rhuIL-4 (0.1–10 μg/well) were coated onto 96-well microtiter plates in 0.1 mL of 50 mM sodium phosphate, pH 8.0, for 18 h at 4 °C. The wells were blocked with 1% BSA in 50 mM sodium phosphate buffer, pH 8.0, for 1 h. Four washes were performed with PBS containing 0.1% Tween 20, followed by the addition of the anti-IL-4 monoclonal antibody (0.145 μg/well) in blocking buffer. After a 1-h incubation at 25 °C followed by four washes, 0.2 mL of a 1:10 000 dilution of horseradish peroxidase conjugated goat anti-rat or anti-mouse IgG (Calbiochem) in blocking buffer was added. After an additional 45-min incubation and four washes, 0.2 mL of a freshly prepared solution of substrate (0.5 mg/mL ABTS in 0.1 M citrate–phosphate, pH 4.0, containing 0.003% H_2O_2) was added. Absorption at 405 nm was determined by a Titertek Multiskan MCC/340 ELISA reader (Flow Laboratories, McLean, VA) after the solution was incubated for 20 min with substrate. Nonspecific binding was determined simultaneously in separate wells by including 10 μg of rhuIL-4 in the anti-IL-4 antibody solution.

Radioimmunoassay. Determination of the binding affinity of radiolabeled rhuIL-4 to monoclonal antibodies and competition studies with synthetic peptides were accomplished by radioimmunoassay. For binding affinity determination, a constant amount (0.1 pmol) of antibody was incubated with increasing concentrations of ^{125}I-rhuIL-4 (2–200 × 10^4 cpm, specific radioactivity of 1500 Ci/mmol) in a final volume of 0.115 mL of RPMI 1640 containing 10% FBS. After a 1-h incubation at room temperature, the antigen–antibody complex was adsorbed to a large excess of Omnisorb cells (0.025 mL of a 10% solution) and centrifuged through an oil gradient (1:1 ratio of dibutyl phthalate to dioctyl phthalate) in a microcentrifuge tube. The tube was rapidly frozen in liquid nitrogen; the tip containing the cell pellet was placed in a vial for determination of radioactivity. Nonspecific binding was determined for each concentration point by adding an excess (60 pmol) of unlabeled rhuIL-4. Nonspecific binding to an appropriate antibody isotype control was also determined. K_d values were determined from a Scatchard analysis using the LIGAND program (Munson & Rodbard, 1980). The number of binding sites per molecule was derived from the known antibody concentration for comparison with the theoretical value for IgG.

In competition studies with IL-4 synthetic peptides, a constant amount of antibody (0.1–0.7 pmol) and ^{125}I-rhuIL-4 (10 000–20 000 cpm) were incubated with varying concentrations of peptides for 1 h at room temperature in the same binding buffer described above. A plate capture method was first developed for studies with monoclonal antibody 11B4. Microtiter plates were precoated with 80 μg/well goat anti-rat γ-globulin (Cappel Labs, Malvern, PA) in 0.1 mL of 50 mM Tris·HCl and 50 mM NaCl, pH 7.4, for 18 h at 4 °C. The wells were subsequently blocked with 1% BSA for 1 h at room temperature and washed with 0.05% Tween 20 in 50 mM Tris·HCl and 50 mM NaCl, pH 7.4, before use. Complete capture of the 11B4 antibody–^{125}I-rhuIL-4 complex onto the microtiter plate was achieved after a 1-h incubation at room temperature. For determination of bound radioactivity, each well was washed three times with 0.2 mL of 0.05% Tween 20, followed by elution with 0.3 mL of 1.0 N NaOH for 1 h at room temperature. The eluates were then transferred to vials for radioactivity measurements. In competition studies with the antibodies 25D2, 35F2, and 1A2, the bound ^{125}I-rhuIL-4 was separated from the free form by adsorption to Omnisorb as described in the procedure for determination of the binding constant. This latter method required fewer manipulations while remaining as effective as the plate capture method. Unlabeled rhuIL-4 was used as a positive control in all displacement analyses.

Mutagenesis. Random and site-specific mutageneses of amino acids 79–96 of rhuIL-4 were performed by cloning

double-stranded oligonucleotide cassettes between the unique *Pvu*II (cuts between Q78 and L79) and *Eco*RI (cuts after L96) sites in the rhuIL-4 coding region. Random substitutions were accomplished throughout each strand during oligonucleotide synthesis by using "doped" nucleotide pools (Hermes et al., 1989). Each mononucleotide pool contained 1.67% of each of the remaining three mononucleotides. This mixture ratio results in a 5% probability that an incorrect nucleotide would replace the specified nucleotides in a specific codon in the sequence. Complementary oligonucleotides were kinased and annealed as described (Maniatis et al., 1982). Annealed cassettes were cloned into a periplasmic expression vector similar to pKGT269-2, as described (Lundell et al., 1990). Mutant rhuIL-4 sequences were identified by DNA sequencing of the relevant area of the plasmid.

Purification and Analysis of RhuIL-4 Mutant Proteins. Individual mutant clones were fermented in 500-mL volumes (Lundell et al., 1990). Using this expression system, rhuIL-4 protein secreted into the periplasm was also released into the culture medium. After the cells were removed by centrifugation, the culture supernatant was chilled on ice and the pH was adjusted to 4.0 with 1 N HCl, followed by addition of 50% TCA to pH 3.0. The mixture was further incubated for 1 h on ice, followed by centrifugation. The pellet was redissolved in 50 mL of 10 mM Tris·HCl, pH 8.0, containing 1% (w/v) CHAPS (Sigma, St. Louis, MO). The protein was purified by batch adsorption onto 5 mL of SP-Sephadex (Pharmacia, Piscataway, NJ) preequilibrated in the same buffer at 4 °C. After an overnight incubation period the resin was packed into a chromatography column, washed with 10 mM Tris·HCl, pH 8.0, and 0.1 M NaCl, and eluted with 10 mM Tris·HCl, pH 8.0, and 1.0 M NaCl. Purity of the eluted rhuIL-4 proteins was ascertained by SDS–PAGE and Coomassie Blue staining. Biological activity was determined using the T-cell proliferation assay described below. [^{3}H]Thymidine incorporation due to the activity of the mutant proteins was compared to that due to wild-type rhuIL-4. The relative receptor binding activity of mutant proteins was similarly compared to wild-type rhuIL-4 in the receptor binding assay described below.

Neutralization Assays. The neutralizing activity of the monoclonal antibodies was determined in a receptor assay. Various concentrations of highly purified antibodies were preincubated with 33 pM ^{125}I-rhuIL-4 for 18 h at 4 °C, followed by the addition of 2×10^6 Daudi cells for each aliquot. All subsequent steps were performed as previously described (Le et al., 1991). In parallel, the neutralization effect of the monoclonal antibodies was also determined in a T-cell proliferation assay. Peripheral blood mononuclear cells were isolated on a Ficoll–Hypaque gradient and cultured in RPMI 1640 medium containing 10% human AB serum, 50 μM 2-mercaptoethanol, and 20 μg/mL PHA. After 4–6-day stimulation the PBL-blasts were harvested for proliferation assay. RhuIL-4 (2.0 ng) and varying amounts of monoclonal antibodies, or appropriate isotype controls, were preincubated for 1 h at 37 °C in microtiter wells containing 0.1 mL of medium per well. PBL-blasts (2×10^4 cells in 0.05 mL) were added to this mixture, and the final volume was brought up to 0.2 mL with culture medium. The plates were incubated for 2 days at 37 °C in a CO_2 incubator. [^{3}H]Thymidine (20 μL containing 1 μCi) was added to each well, and the cells were incubated for 18 h at 37 °C. Cells were harvested on glass fiber filters, washed with water and methanol, and allowed to dry before addition of scintillation fluid for radioactivity determination. Cell proliferation was assessed by the measurement of [^{3}H]thymidine incorporated during the last 18 h of culture. Neutralization was expressed as the molar concentration (IC_{50}) of antibody that decreases by 50% the receptor binding or T-cell proliferation activity of rhuIL-4.

Table I: Apparent Dissociation Constants and Neutralization Titers of Monoclonal Antibodies Raised to RhuIL-4[a]

monoclonal antibody	species/ isotype	app K_d (nM)	IC_{50} (nM) T-cell proliferation assay	IC_{50} (nM) receptor binding assay
11B4	rat/IgG_{2a}	0.43	2.0	2.0
25D2	rat/IgG_1	2.9	0.33	0.33
35F2	rat/IgG_{2a}	2.4	0.79	0.27
1A2	mouse/IgG_1	50	nonneutralizing	

[a] The apparent dissociation constant (K_d) was estimated by Scatchard analysis of the binding of monoclonal antibody to ^{125}I-rhuIL-4. Determination of bound and free ^{125}I-rhuIL-4 was performed as described under Experimental Procedures. The calculated number of binding sites per antibody molecule was 2.1, 2.0, 1.9, and 2.2 for antibodies 11B4, 25D2, 35F2, and 1A2, respectively. Methods for the determination of T-cell proliferation and receptor binding activities are described under Experimental Procedures.

Electrophoresis. A modification of the procedure of Laemmli (1970) was employed for discontinuous SDS–PAGE on 20% slab gels. Immunoblots with anti-IL-4 monoclonal antibodies were performed as described by Towbin et al. (1979). The blots were incubated with the monoclonal antibody solutions (1.0 μg/mL) in 50 mM sodium phosphate, pH 8.0, containing 1% gelatin for 18 h at room temperature. For detection of bound antibodies 11B4, 25D2, and 35F2, the second antibody was horseradish peroxidase conjugated goat anti-rat IgG. The second antibody for detection of bound antibody 1A2 was horseradish peroxidase conjugated goat anti-mouse IgG. The substrate for the peroxidase reaction was 4-chloro-1-naphthol.

Protein Determination. The concentration of rhuIL-4 was determined spectrophotometrically using the extinction coefficient $E^{0.1\%}_{278\ nm} = 0.625\ mg^{-1}\ cm^2$ (Windsor et al., 1991). The concentration of the anti-IL-4 monoclonal antibodies was also estimated spectrophotometrically using the extinction coefficient $E^{0.1\%}_{278\ nm} = 1.35\ mg^{-1}\ cm^2$ (Little & Donahue, 1967).

RESULTS

Binding Affinity and Neutralization Activity of Monoclonal Antibodies to RhuIL-4. Four monoclonal antibodies to rhuIL-4 were studied for their binding affinity to radiolabeled rhuIL-4 and for their neutralization activity in a receptor binding assay and a T-cell proliferation assay (Table I). Rat antibody 11B4 exhibited high binding affinity for ^{125}I-rhuIL-4 with an apparent K_d in the subnanomolar range (K_d = 0.43 nM). The K_d value estimated for 11B4 was notably 5–6-fold lower than the apparent K_d values observed for the other two rat monoclonal antibodies, 25D2 and 35F2. All three rat monoclonal antibodies bound to ^{125}I-rhuIL-4 with higher affinity than the mouse monoclonal antibody 1A2 (K_d = 50 nM). Antibodies 11B4, 25D2, and 35F2 were all neutralizing in either receptor binding assay or T-cell proliferation assay in nanomolar or subnanomolar concentrations, whereas 1A2 was not neutralizing in these assays. This result is consistent with the previously reported neutralizing activity of antibody 11B4 (Chretien et al., 1989). Interestingly, the relative potency of neutralization of 11B4 was not proportional to its apparent binding affinity for ^{125}I-rhuIL-4. Thus, antibody 11B4, which exhibited the highest affinity for ^{125}I-rhuIL-4,

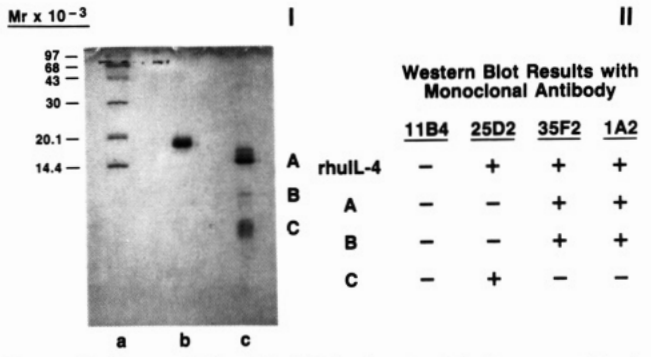

FIGURE 1: Immunoblots of rhuIL-4 and proteolytic fragments. Panel I: rhuIL-4 and the fragments obtained by selective proteolytic cleavage with *S. aureau* V8 protease were subjected to discontinuous SDS–PAGE on a 20% polyacrylamide slab gel; the proteins were stained by Coomassie brilliant blue R250. Lanes: (a) molecular weight standards (phosphorylase *b*, 97K; bovine serum albumin 68K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 21.5K; lysozyme, 14.4K); (b) rhuIL-4; (c) *S. aureus* protease digest of rhuIL-4. Digestion of rhuIL-4 with *S. aureus* V8 and identification of the resulting fragments were performed as described under Experimental Procedures. Fragments A and B represent huIL-4 residues 27–103 and 44–103, respectively. Fragment C was a mixture of huIL-4 residues 1–19 and 104–129. Panel II: binding of monoclonal antibodies to the Western-blotted proteins and proteolytic fragments was performed as described under Experimental Procedures. A plus sign (+) represents an unambiguous immunostaining of the band with a relative intensity comparable to the Coomassie blue staining pattern; a minus sign (–) represents no staining or trace staining of an ambiguous nature.

was not as potent as either antibody 25D2 or 35F2 in both neutralization assays. Antibodies 25D2 and 35F2 exhibited nearly identical binding affinities for ^{125}I-rhuIL-4 as evidenced by K_d values of 2.9 and 2.4 nM, respectively. Similarly, the ability of these two monoclonal antibodies to neutralize either the binding of ^{125}I-rhuIL-4 or the bioactivity of rhuIL-4 was approximately equivalent. In studies with antibody 1A2 in the T-cell proliferation assay and, in particular, in the receptor binding assay, neutralization could not be demonstrated at concentrations exceeding the K_d concentration by 100-fold (data not shown). The lack of neutralization apparently did not result from the relatively lower affinity of this antibody for rhuIL-4.

Binding of Monoclonal Antibodies to Proteolytic Fragments of RhuIL-4. Proteolytic fragments of rhuIL-4 were obtained by *S. aureus* V8 protease digestion, as previously described (Le et al., 1991); after reduction with 2-mercaptoethanol the digest was subjected to SDS–PAGE. Western blots of the separated fragments were probed for binding to each of the four monoclonal antibodies (Figure 1). Antibody 11B4 failed to bind to all fragments, including the full-length reduced form of rhuIL-4. The latter observations are consistent with previous findings that antibody 11B4 only binds significantly to the nonreduced form of rhuIL-4 in Western blots (Chretien et al., 1989). Antibody 25D2 bound equally well to reduced rhuIL-4 and band C, which had been shown to be composed of a mixture of two huIL-4 peptides, residues 1–19 and 104–129 (Le et al., 1991). Both antibodies 35F2 and 1A2 bound to reduced rhuIL-4 and bands A and B, but failed to recognize band C. Bands A and B were identified as residues 27–103 and 44–103.

Binding of Monoclonal Antibodies to Synthetic Peptides. Binding of the four monoclonal antibodies to these peptides was evaluated by radioimmunoassay and by direct ELISA. Antibody 11B4 was tested for binding to six synthetic peptides comprising the majority of the amino acid sequence of rhuIL-4, i.e., residues 32–39, 43–59, 61–82, 65–82, 83–104, and

Table II: Binding of Monoclonal Antibodies to HuIL-4 Synthetic Peptides in Radioimmunoassay and ELISA[a]

	monoclonal antibody			
huIL-4 peptide	11B4	25D2	35F2	1A2
1–26		–	–	–
32–39	–	–	–	–
43–59	±		–	+
52–65			–	
61–82	+		–	–
65–82	–			
70–92			+	
83–104	–		–	–
97–117			–	–
99–111		–		
104–112		–		
104–129	–	+	–	–
113–120		–		
121–126		–		
rhuIL-4 (20–103)[b]		–	+	+

[a] The monoclonal antibodies were tested for binding to peptides representing various overlapping segments of huIL-4 in ELISA and in competitive radioimmunoassay using ^{125}I-rhuIL-4. In ELISA studies, 0.1–10 μg of peptides in 0.1 mL of 50 mM sodium phosphate, pH 8.0, was used to coat the wells of the microtiter plates. In radioimmunoassay studies, an increasing concentration of peptide up to 10 μM was used to displace the binding of 20 000 cpm of ^{125}I-rhuIL-4 to 0.1 pmol of antibody. Details of these assays are described under Experimental Procedures. A plus sign (+) indicates binding as measured by ELISA signals ($A_{405nm} \geq 0.05$) significantly above background ($A_{405nm} = 0.01$) and by the inhibition (≥50%) of binding of ^{125}I-rhuIL-4 to the antibody by 10 μM peptides. A minus sign (–) indicates a lack of binding in both assays by the above criteria. (±) refers to binding observed by ELISA although no displacement of ^{125}I-rhuIL-4 binding to the antibody was observed in radioimmunoassay. [b] RhuIL-4 (20–103) is a large proteolytic fragment obtained by *S. aureus* V8 digestion of rhuIL-4 and purified by reversed-phase HPLC as described previously (Le et al., 1991).

104–129. Unambiguous binding was observed only for peptide 61–82 in radioimmunoassay. Interestingly, 11B4 failed to recognize synthetic peptide 65–82, which differs from peptide 61–82 by only four residues truncated from the N-terminus. Studies with monoclonal antibody 25D2 confirm the Western blot analysis of rhuIL-4 fragments obtained by *S. aureus* V8 digestion (Figure 1). 25D2 failed to bind to the core fragment, residues 20–103, in both radioimmunoassay and ELISA studies. Furthermore, testing of a series of huIL-4 synthetic peptides comprising residues 1–26, 32–39, 99–111, 104–112, 104–129, 113–120, and 121–126 showed that 25D2 exhibited binding affinity for a single peptide, residues 104–129, representing the C-terminal sequence. Testing of 35F2 and 1A2 for binding to the panel of synthetic peptides shown in Table II revealed that the two antibodies recognized two distinct sequences, residues 70–92 and 43–59, respectively. As expected, both antibodies recognized the large core fragment obtained by *S. aureus* V8 digestion spanning residues 20–103 of rhuIL-4.

The concentration dependence of the binding displacement of ^{125}I-rhuIL-4 by huIL-4 synthetic peptides 61–82, 104–129, 70–92, and 43–59 for the corresponding monoclonal antibody was examined (Figure 2). The studies performed by radioimmunoassay compared the binding displacement of synthetic peptide and unlabeled native rhuIL-4 for each monoclonal antibody. Inhibition of binding of ^{125}I-rhuIL-4 to antibody 11B4 required an exceedingly high concentration of rhuIL-4 peptide 61–82, in excess of 1 μM (Table III). The calculated K_i of inhibition (i.e., 1.87 μM) was approximated 4000-fold greater than the calculated K_i for unlabeled rhuIL-4. Studies with antibody 25D2 showed that the K_i for peptide 104–129 was 196 nM and, hence, was approximately 50-fold greater than the K_i for unlabeled rhuIL-4 (Figure 2B and

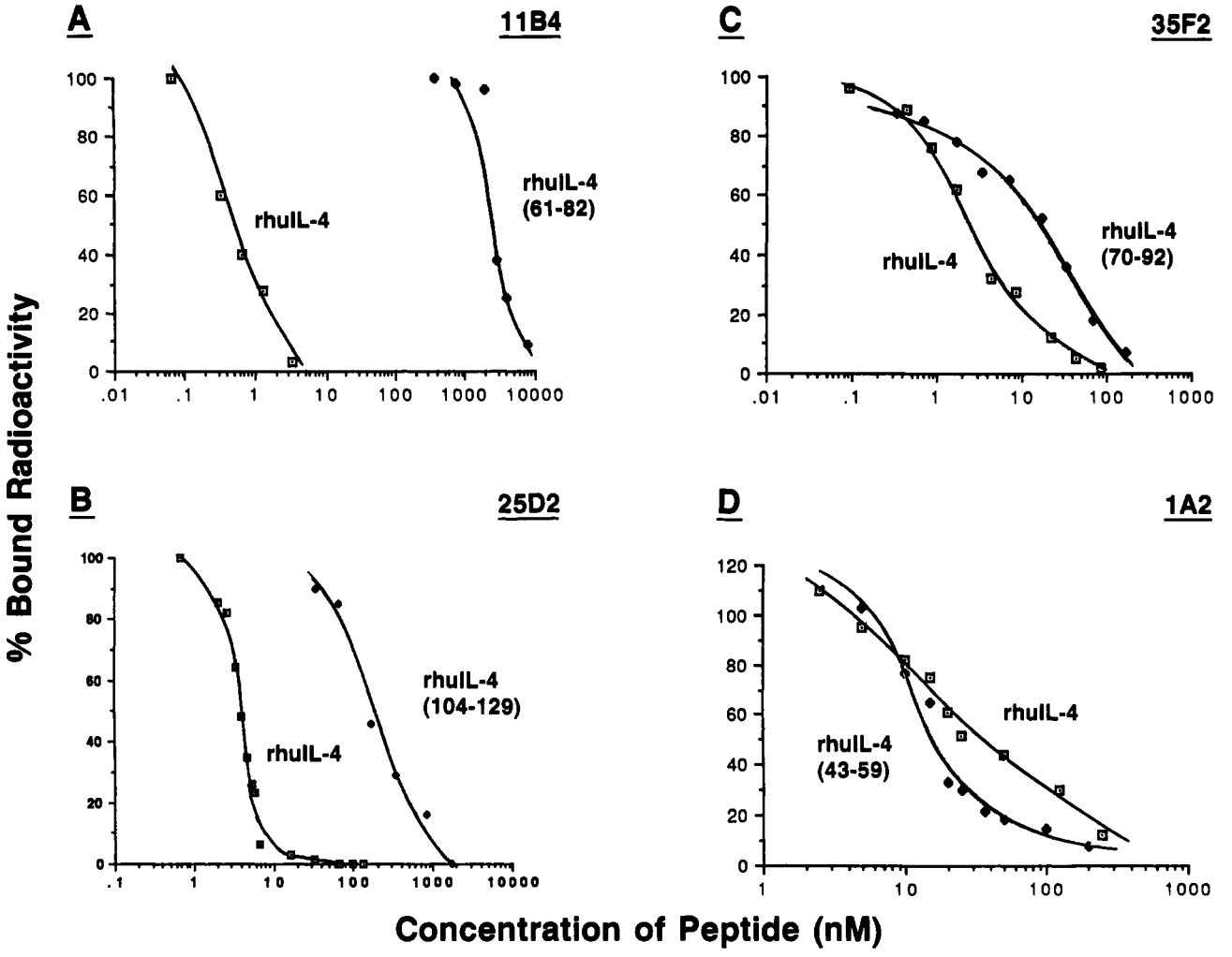

FIGURE 2: Inhibition of ^{125}I-rhuIL-4 binding to monoclonal antibodies by synthetic peptides. Binding displacement studies with synthetic huIL-4 peptides 61–82, 104–129, 70–92, and 43–59 for monoclonal antibodies 11B4 (panel A), 25D2 (panel B), 35F2 (panel C), and 1A2 (panel D), respectively, were performed as described under Experimental Procedures.

Table III: K_i Values for the Inhibition of ^{125}I-rhuIL-4 Binding to Monoclonal Antibodies by Unlabeled RhuIL-4 and Synthetic Peptides[a]

	K_i (nM)				
monoclonal antibody	unlabeled rhuIL-4	rhuIL-4 synthetic peptides			
		43–59	61–82	70–92	104–129
1A2	30	13			
11B4	0.47		1869		
35F2	1.96			19.6	
25D2	3.93				196

[a] K_i values for the unlabeled rhuIL-4 and synthetic peptides were derived from the corresponding IC_{50} data using the equation $K_i = IC_{50}/(1 + L/K_d)$ of Cheng and Prussoff (1973), where L is the concentration of ^{125}I-rhuIL-4 and K_d is the apparent dissociation constant for the binding of ^{125}I-rhuIL-4 to the antibody. The IC_{50} values were deduced from the experiments shown in Figure 2.

Table III). The difference in K_i for peptide 70–92 and unlabeled rhuIL-4 in competition studies with antibody 35F2 was noticeably less. However, the observed K_i value for peptide 70–92 (i.e., 19.6 nM) was 10-fold greater than the K_i of unlabeled rhuIL-4. Nonneutralizing antibody 1A2 was the only antibody capable of binding a rhuIL-4 synthetic peptide, residues 43–59, with an affinity comparable to that for unlabeled rhuIL-4 (Figure 2D). The observed K_i values for peptide 43–59 and rhuIL-4 were 13 nM and 30 nM, respectively (Table III).

Mutagenesis of Selected Residues in RhuIL-4. The importance of amino acid residues in the rhuIL-4 sequence 70–92, which was recognized by the neutralizing antibody 35F2, was analyzed in mutagenesis studies. As shown in Table IV, amino acid residues in the hydrophilic cluster K84-R85-L86-D87-R88-N89 and adjacent residues were targeted for mutagenesis studies. Of special interest were the charged residues K84, R85, D87, and R88 that form part of the hydrophilic surface of the amphipathic helix comprising residues 70–93 (Walter et al., 1992; Redfield et al., 1991). Substitution of uncharged residues for charged residues, or reversing the polarity of the charged residues, caused the most significant changes in biological activities. K84, D87, and R88 were the most sensitive to these substitutions. Complete loss of both T-cell proliferation and receptor binding activity was observed for mutants K84I, D87G, R88Q, and R88T and for the double mutant R88T, N89D. Substitution of serine and glutamine for arginine at positions 81 and 85 did not strongly affect biological activities, as evidenced by the results obtained for mutant R85Q and the double mutant R81S, R85Q. Substitution of histidine for arginine at residue 88 did not totally abolish activity, in contrast to the abrogation that resulted from substitution with uncharged residues at the same position (mutants R88Q and R88T).

An additional noteworthy effect of substitution was observed at N89, a residue which is positioned toward the hydrophobic surface of amphipathic helix 70–93 (Walter et al., 1992).

Table IV: Bioactivity and Receptor Binding of Selected rhuIL-4 Mutants[a]

rhuIL-4 mutant	T-cell proliferation activity (%)	receptor binding activity (%)
F82A	100	100
F82S	100	100
K84I	0	1
R85Q	50	50
D87G	0	nd
R88H	50	26
R88Q	3	5
R88T	0	0
N89D	10	1
N89S	90	60
W91L	100	100
G92D	100	100
R81S, R85Q	50	10
R88T, N89D	0	0
F82Y, K84R, L90V	100	100
wild type	100	100

[a] The nomenclature for describing substitution mutants is as follows: a one-letter code to indicate the substituted amino acid in the wild-type sequence, followed by a number to indicate its position, followed by a one-letter code to indicate the amino acid substituted in the mutant. The *E. coli*-derived rhuIL-4 and the mutants were purified from culture supernatants to a high degree of homogeneity (90–95% pure, as estimated by SDS–PAGE) by the method described under Experimental Procedures. Their specific activities in the T-cell proliferation assay were determined and expressed as a percentage of the wild-type activity (2×10^7 units/mg). The receptor binding studies were performed with U-937 and ^{125}I-rhuIL-4. U937 cells (2×10^7 cells/mL) were incubated in 1 mL of RPMI 1640 containing 10% FBS and 33 pM ^{125}I-rhuIL-4. The binding displacement of *E. coli*-derived wild-type rhuIL-4 or mutants was determined by adding 2 μg/mL (130 nM) purified proteins which, in the case of the wild type, was sufficient to cause displacement of specifically bound radioactivity to background level. The activity of the mutants was expressed as a percentage of binding displacement of the wild type.

Substitution causing charge reversal (mutant N89D) resulted in a 10–100-fold decrease in activity, whereas substitution with an uncharged hydrophilic residue (mutant N89S) did not significantly alter activity. Mutations that did not appear to affect the biological activities of rhuIL-4 were observed at F82, W91, and G92. The high level of biological activity obtained with the triple mutant F82Y, K84R, L90V indicated that conservative substitutions at K84 and L90 did not significantly affect biological activities.

Location of Epitopes in the Three-Dimensional Structure of RhuIL-4. The X-ray crystallographic determination of the three-dimensional structure of rhuIL-4 has recently been determined at 2.5-Å resolution (Walter et al., 1992). As shown in Figure 3, epitope mapping indicates that the major structural elements in rhuIL-4 that interact with the neutralizing antibodies are the BC loop (residues 60–69), helix C (residues 70–93), β-strand 2 (residues 105–108), and helix D (residues 109–127). Although these structures are clustered toward the C-terminal end in the primary structure, they are distinctly apart in the three-dimensional structure. The up–up–down–down topology of the α-helices in rhuIL-4 places helices A and B and helices C and D across from each other rather than next to each other. Thus, the binding sites for antibodies 35F2 (helix C) and 11B4 (BC loop and part of helix C) are distinctly segregated from the binding site of antibody 25D2 (β-strand 2 and helix D).

DISCUSSION

The studies presented here were designed to identify the epitopes recognized by three neutralizing monoclonal antibodies and one nonneutralizing antibody directed to rhuIL-4 in order to identify potential sites for interaction of rhuIL-4 with its receptor. Of special interest is the fact that the neutralizing antibodies recognized two spatially distinct domains in the C-terminus of rhuIL-4. The epitope for the nonneutralizing antibody was mapped to a sequence in the B helix that is distinct from either of these domains. Each of the antibodies that neutralized biological activity was shown to inhibit binding of rhuIL-4 to its cell-surface receptor with an IC_{50} comparable to that observed for in vitro inhibition of biological activity. Thus, it is reasonable that the sites recognized by the three neutralizing antibodies are present at or near receptor binding regions. On the basis of the high thermodynamic stability of rhuIL-4 (Windsor et al., 1991) and its relatively rigid four α-helical bundle structure stabilized by three disulfide bonds (Walter et al., 1992), it appears less likely that neutralization of biological activity was a consequence of a large conformation change. It is notable that a polyclonal antibody that recognizes rhuIL-4 (Le et al., 1991) as well as the monoclonal antibody 1A2 that is described here binds to rhuIL-4 without neutralizing biological activity.

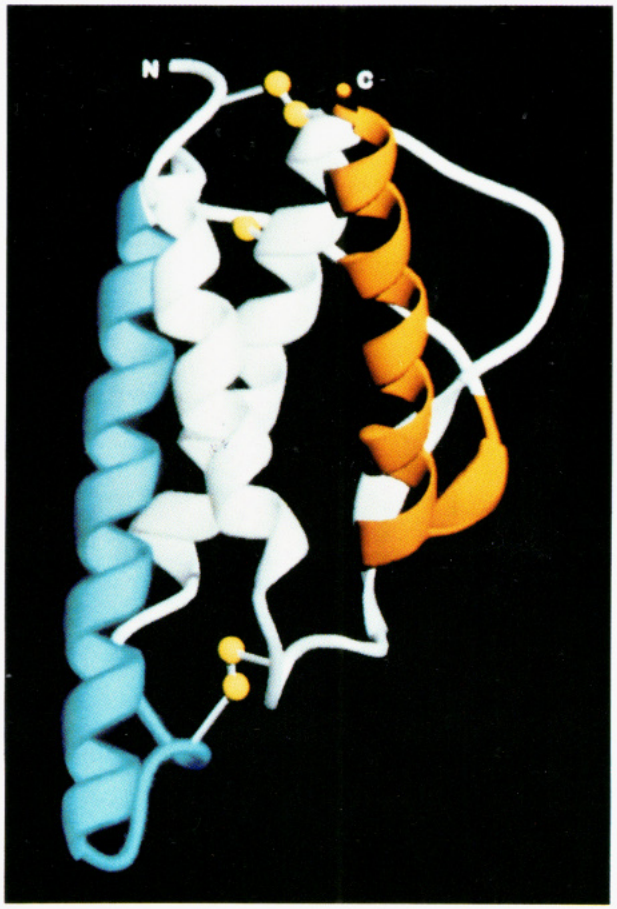

FIGURE 3: Binding epitopes of the neutralizing monoclonal antibodies 11B4, 25D2, and 35F2 in the three-dimensional structure of rhuIL-4. The three-dimensional structure of rhuIL-4 is represented as a ribbon drawing based on the C_α position. The binding epitopes for antibodies 11B4 and 35F2 are colored blue, and the binding epitope for antibody 25D2 is indicated in orange. The sulfur atoms in the disulfide bonds are represented as yellow spheres. The program described by Carson (1987) was used in the preparation of the figure.

Mapping of the epitopes was accomplished by a combination of Western blot analysis of rhuIL-4 proteolytic fragments, direct ELISA, and radioimmunoassays with a panel of synthetic peptides. The pattern of binding of the antibodies to large proteolytic fragments on Western blots provided the initial leads for selecting appropriate huIL-4 peptides for ELISA binding and competitive radioimmunoassay studies. The competitive radioimmunoassays were essential for de-

termining the relative binding affinity of each antibody for the corresponding peptides and native rhuIL-4.

The lack of significant binding of 11B4 to the reduced form of rhuIL-4 in Western Blots suggested that this antibody recognized a conformational epitope highly dependent upon the integrity of disulfide bonds. This observation was consistent with subsequent studies with 11B4 and synthetic peptides. A weak ELISA response was detected for huIL-4 peptide 61–82, and similarly a large K_i value (1869 nM) was observed in competitive radioimmunoassay studies with the same peptide. The data suggested that 11B4 recognizes certain structural elements in peptide 61–82 that are also present in native rhuIL-4. The apparent requirement for residues 61–64 (KDTR) for binding of peptide 61–82 to antibody 11B4 was particularly notable. Plausibly, these four N-terminal residues could stabilize the peptide in a favorable conformation for recognition by antibody 11B4 or could contribute directly to binding. It is of interest that, in competitive radioimmunoassay studies with radiolabeled rhuIL-4, antibodies 35F2 and 25D2 exhibited K_i values of 19.6 nM and 196 nM for huIL-4 peptides 70–92 and 104–129, respectively. These values were within an order of magnitude of the K_i value determined for competition with unlabeled rhuIL-4. Notably, both huIL-4 peptides 70–92 and 104–129 exhibited α-helical structures in 20 mM sodium phosphate at pH 7.2, as determined by circular dichroism (W. Windsor, unpublished observation). From the available data the epitopes for 35F2 and 25D2 could reside entirely within residues 70–92 and 104–129, respectively. The observed higher K_i values might be due to the fact that the synthetic peptides in solution could assume conformations different from that found in the cognate sequences in native rhuIL-4. Alternatively, 35F2 and 25D2 could recognize other minor epitopes in huIL-4 that escape detection by the methodology employed in this study. Interestingly, neither peptide 61–82 nor peptide 83–104, which contain sequences derived from the N- and C-terminal portions of peptide 70–92, competed with antibody 35F2 for binding to radiolabeled rhuIL-4. Thus, the epitope for antibody 35F2 is likely to reside in an amino acid sequence derived from at least a portion of each of these two sequences.

The C-terminal sequence in rhuIL-4 has been previously implicated as an important part of the receptor binding domain in studies from our laboratory on the biological activity of large proteolytic fragments and the neutralization properties of antisera directed to the N- and C-termini (Le et al., 1991). Similarly, the C-terminal sequence of murine interleukin 4 has also been implicated in receptor binding in mutagenesis studies reported by Morrison and Leder (1992). The present epitope assignment for neutralizing monoclonal antibody 25D2 to residues within the C-terminal sequence 104–129 lends further support to these findings. In addition, these data are in agreement with the model proposed by Bazan (1990) that exposed residues in the C-terminal helix (helix D) in helical cytokines form a key receptor recognition motif. Experimental evidence supporting a receptor binding function for residues located in the domain recognized by antibody 35F2 (helix C) was provided, in part, by site-directed mutagenesis. Four residues located within helix C (K84, D87, R88, and N89) were highly sensitive to mutations. In particular, substitution of K84 and R88, which are solvent-exposed residues (Walter et al., 1992), with uncharged and nonhelix breaker residues caused a total loss of biological activity and receptor binding. The retention of at least a portion of biological activity and receptor binding activity in mutant R88H further supports the necessity for at least a partial positive charge at residue 88 in order to achieve receptor interaction. The β-carboxamide moiety of N89 is also solvent exposed and could conceivably be available for receptor interaction through hydrogen bonding. The role of D87 is less obvious since substitution with glycine (a helix breaker) could result in substantial distortion of helix C, thus perturbing the overall structure of rhuIL-4. It is interesting to note that, in addition to our data supporting a potential role for helix C in receptor interaction, Postlethwaithe and Seyer (1991) have shown that the synthetic peptide corresponding to residues 65–98 in rhuIL-4 induced chemotaxis in human fibroblasts, although a significantly higher concentration than rhuIL-4 was required.

Recent X-ray crystallographic studies of the human growth hormone and receptor complex have shown that amino acid residues from two nonneighboring helices could very well be involved in receptor binding (de Vos et al., 1992; Cunningham et al., 1991). Such interaction occurred in a complex consisting of two molecules of receptor binding one molecule of growth hormone. A similar model of interaction for rhuIL-4 and its receptor could account for the neutralization activity of antibodies 35F2, 25D2, and 11B4 and is consistent with the classification of rhuIL-4 and growth hormone receptors as members of the class I cytokine receptor superfamily (Bazan, 1990; Cosman et al., 1990). Binding of the antibodies to a site occupied by either one of the two receptors would block the formation of the high-affinity ternary complex and potentially inhibit subsequent signal transduction events. Confirmation of the model, however, must await biophysical studies of the high-affinity complex formed between the extracellular domains of the recombinant receptor and rhuIL-4.

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