A Murine Interleukin-4 Antagonistic Mutant Protein Completely Inhibits Interleukin-4-induced Cell Proliferation, Differentiation, and Signal Transduction*

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We characterize here a highly efficient antagonist for interleukin-4 (IL-4) in the mouse system. In this double mutant of the murine IL-4 protein, both glutamine 116 and tyrosine 119 were substituted by aspartic acid residues. This variant (QY) bound with similar affinity to the IL-4 receptor β subunit as wild type IL-4 without inducing cellular responses. In contrast, QY completely inhibited in a dose-dependent manner the IL-4-induced proliferation of lipopolysaccharide-stimulated murine splenic B-cells, of the murine T cell line CTLL-2, and of the murine pre-B-cell line BA/F3. QY also inhibited the IL-4-stimulated up-regulation of CD23 expression by lipopolysaccharide-stimulated murine splenic B-cells and abolished tyrosine phosphorylation of the transcription factor Stat6 and the tyrosine kinase Jak3 in IL-4-stimulated BA/F3 cells. Selective inhibition of IL-4 may be beneficial in T-helper cell type 2-dominated diseases, like type 1 hypersensitivity reactions or helminthic infections. The QY mutant could be an attractive tool to study in vivo the therapeutic potential of IL-4 antagonists in mouse systems.

Interleukin-4 (IL-4)

Interleukin-4 (IL-4) is a pleiotropic cytokine derived from T-cells, thymocytes, and mast cells that has multiple effects on many cell types (1). Its functions include the differentiation of T helper cells to a Th2 phenotype and the induction of sterile γ transcripts in B-cells, which is a required step for class switching to IgE type antibodies (2, 3). Among the clinically important features of IL-4 are the coordination of immune responses against helminthic macroparasites and its central role in the sensitization process of type I allergic diseases (4, 5).

Two receptor proteins have been identified for IL-4. The 140-kDa IL-4 receptor α chain (IL-4Ra) binds IL-4 with high affinity (6–8). The second subunit is the 64-kDa common γ receptor subunit (γc) (9, 10), which is also shared by the receptor for IL-2, IL-7, IL-9, and IL-15 (11).

Site-directed mutagenesis has led to the discovery of two regions in the human IL-4 molecule that are important for interaction with the receptor chains (for review see Ref. 12). Substitution of glutamic acid 9 (Glu9) or arginine 88 (Arg88) leads to a near complete loss of binding to IL-4Ra (13). A second important region of the human IL-4 molecule is defined by mutations of arginine 121, tyrosine 124, and serine 125, close to the C terminus of the protein. Mutations at these positions do not interfere with IL-4Ra binding but can severely impair cellular responses, because they result in loss of interaction with γc (14). The most efficient single site mutant of this type is Y124D, which does not induce T-cell proliferation but is a partial agonist for up-regulation of CD23 on B-cells (15). In contrast, a double mutant where both Arg121 and Tyr124 have been replaced by aspartic acid (RY) has no agonistic activity in any assay employed so far (16). RY is therefore a perfect high affinity antagonist for human IL-4, as well as for IL-13, which also uses IL-4Ra for signal transduction (16, 17).

IL-4 antagonists may provide an effective way for the therapy of Th2-dominated diseases. The major obstacle for testing in vivo biological tolerance and therapeutic effects of IL-4 antagonists is the species specificity of IL-4. There is approximately 60% DNA sequence homology (18, 19) and no cross-reactivity between human and mouse IL-4 (20). Due to the lack of receptor binding, human IL-4 antagonists cannot be effective in mice. This has prompted us to develop an efficient antagonist for IL-4 in the mouse system.

MATERIALS AND METHODS

Animals, Cells, and Viruses—BALB/c mice 6–8 weeks of age were purchased from Charles River/Wiga. The Spodoptera frugiperda insect cell line Sf9 was cultured in Insect Xpress medium (Serva, Heidelberg, Germany) without adjuvants at 27 °C in a 2-liter spinner flask aerated with 100% oxygen. Autographa californica nuclear polyhedrosis virus DNA (BaculoGold™DNA) was from Pharmingen (Hamburg, Germany).

Murine cells were cultured in RPMI 1640 containing 8% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, supplemented with 100 ng/ml IL-2 for CTLL-2 cells and with 5% culture supernatant of murine mIL-3-producing X63Ag8–563 BPV cells (21) for BA/F3 cells.

Production of IL-4 Wild Type and Double Mutant Proteins—Recombinant IL-4 proteins were produced in Sf9 cells following Baculovirus infection. The pEP-B splice mIL-4 plasmid containing the cDNA for mouse IL-4 was kindly provided by Dr. Werner Müller (University of Cologne, Germany) (22). A fragment encoding the cDNA of mouse IL-4 was amplified by polymerase chain reaction with the synthetic 5′ primer 5′-CCGGATCCATCCATCCGAGGCA-3′ and the 3′ primer 5′-GGCGATCCCTAGCTGTAATC-3′, comprising a BamHI site and the first (for the 5′ primer) and, respectively, last (for the 3′ primer) four codons of wild type IL-4. The QY mutant gene was constructed using the 3′ primer 5′-CCGGATCTAGCTGTAATC-3′, resulting in substitution of both glutamine 116 and tyrosine 119 by aspartic acid. The constructs were sequenced and cloned into the Bacu

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A two times higher concentration of QY compared with IL-4.

Association and dissociation rates for the binding of mouse IL-4 and QY to IL-4-BP, as determined by biosensor experiments. The data are mean values (n = 15) ± standard deviation. The results from two independent experiments (Exp.) are shown for each protein.

|       | Association Rate (k_a) | Dissociation Rate (k_d) | Constant (K_d) |
|-------|------------------------|------------------------|---------------|
|       | 10^6 s⁻¹               | 10^-4 s⁻¹              | M⁻¹ s⁻¹       |
| IL-4  |                        |                        |               |
| Exp. 1| 4.6 ± 0.69 x 10^6      | 1.5 ± 0.36 x 10^-4     | 390           |
| Exp. 2| 5.1 ± 0.77 x 10^6      | 1.9 ± 0.48 x 10^-4     | 340           |
| QY    |                        |                        |               |
| Exp. 1| 2.2 ± 0.37 x 10^6      | 1.8 ± 0.54 x 10^-4     | 820           |
| Exp. 2| 2.3 ± 0.60 x 10^6      | 1.9 ± 0.57 x 10^-4     | 830           |
tive cells a higher dose of inhibitor was needed to block IL-4-induced responses.

The low affinity IgE receptor, CD23, is an IL-4-inducible B-cell differentiation marker. Half-maximal CD23 expression was induced by 19 pM IL-4 (Fig. 3A). Similar to the proliferation assays, there was no detectable activity of the QY variant. QY prevented the IL-4-induced CD23 expression with half-maximal inhibition reached at about 95-fold excess of the mutant (Fig. 3B).

The signal transduction of cytokines involves the activation of various tyrosine kinases and rapid phosphorylation of their substrates. IL-4 stimulates phosphorylation and activation of the transcription factor Stat6 and the tyrosine kinase Jak3 (27). To determine whether the QY mutant could inhibit IL-4-induced signaling, we have measured Stat6 and Jak3 phosphorylation in BA/F3 cells. IL-4 at 1 nM concentration induced tyrosine phosphorylation of both proteins, whereas the same amount of the QY mutant did not stimulate phosphorylation (Fig. 4). A 500-fold excess of the QY variant completely inhibited the IL-4-induced response (Fig. 4).

Antagonistic properties of human IL-4 variants are caused by the deletion of a hydrophobic patch on the surface of the IL-4 molecule and by the introduction of an electrostatic mismatch (28). No local instability is introduced in the protein structure, because main chains structures of antagonistic mutants are identical compared with wild type (29).

No structural data on mouse IL-4 are available, but some mutation studies have been reported. A deletion mutant of murine IL-4 lacking residues 118–120 has impaired activity in a proliferation assay with the T-cell line CT4R but retains high affinity binding to IL-4Rα (30). Such a deletion, however, may alter the protein structure, and inhibition of wild type protein was not tested. In the same study, replacement of Gln116 with alanine had only minor effects on receptor binding and biological activity (30). A murine IL-4 variant with a single site mutation of tyrosine 119 (Y119D) has been mentioned in the literature, but in one report no data were shown (31), and in another one the same mutant induced agonistic effects similar to those of wild type IL-4 in differentiation assays, like MHC II up-regulation on B-cells (32). This is to be expected for the Y119D mutant, because murine and human γc interact in a structurally different way with IL-4. Human IL-4 can productively interact both with human and murine γc, but human γc is most severely affected by mutations in Tyr124 (13), and murine γc is most severely affected by mutations in Arg121 (33). For this reason, and in consideration of the partial agonist activities of human Y124D (13, 15), we decided to create the QY

| Cell type | Mean ED50 pM | Mean ID50 nM | Mean KpM |
|-----------|--------------|--------------|----------|
| BA/F3     | 67 ± 6 (n = 7) | 3 ± 0.7 (n = 6) | 190 ± 150 (n = 6) |
| Splenic B-cell | 16 ± 10 (n = 3) | 37 ± 0.9 (n = 3) | 600 ± 380 (n = 3) |
| CTLL-2    | 9 ± 7.4 (n = 7) | 61 ± 53 (n = 2) | 520 ± 300 (n = 2) |

**Table II**

**Effective and inhibitory doses of IL-4 and QY mutant for proliferation in different cell types**

IL-4 concentration required for half-maximal proliferation (ED50) and QY concentration required for half-maximal inhibition (ID50). The data shown are mean values ± standard deviation.
FIG. 4. IL-4-induced tyrosine phosphorylation. Factor-deprived BA/F3 cells were stimulated for 10 min with cytokines in the indicated concentrations. IL-4-induced tyrosine phosphorylation of Jak3 and Stat6 was shown by immunoprecipitation and Western blotting.

double mutant, as a murine analog to the complete human IL-4 antagonist RY (16). As shown here, the QY mutant was by itself inactive and completely antagonistic for wild type IL-4, in all assays performed.

Like IL-4, IL-13 can also direct IgE class switching in human B-cells (34, 35) but apparently not in the mouse, because IL-4 knockout mice are unable to produce IgE (36, 37). Anti-IL-4 antibodies or soluble IL-4 receptors may not be sufficient to treat for example type I hypersensitivity in humans, because knockout mice are unable to produce IgE (36, 37). Anti-IL-4

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