Pharmacokinetic Study of Anti-Interleukin-6 (IL-6) Therapy With Monoclonal Antibodies: Enhancement of IL-6 Clearance by Cocktails of Anti-IL-6 Antibodies

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The use of inhibiting cytokine-binding-proteins (CBPs) such as soluble cytokine receptors and anticytokine antibodies is considered for the treatment of cytokine-dependent diseases. The pleiotropic cytokine interleukin-6 (IL-6) is a target for immunointervention in numerous pathologic situations, including multiple myeloma, B-cell lymphoma, and rheumatoid arthritis. An antitumor response was obtained in the treatment of a patient with multiple myeloma. A controversial issue is to evaluate whether the carrier effect of the CBPs might limit their efficiency in blocking the target cytokine. We analyzed the pharmacokinetics of radiolabeled IL-6 in mice treated with various combinations of anti-IL-6 antibodies. We show that injection of one or two antibodies led to the stabilization of the cytokine. Conversely, simultaneous treatment with three anti-IL-6 antibodies, binding to three distinct epitopes, induced the rapid uptake of the trimeric immune complexes by the liver and the elimination of IL-6 from the central compartment. The use of cocktails of three antibodies binding simultaneously to a cytokine thus provides a new means of enhancing the clearance of the target molecule and should help in the design of antibody-based clinical trials by overcoming the problem of the accumulation of the cytokine in the form of monomeric immune complexes.

MATERIALS AND METHODS

Antibodies. The MoAbs AH64 (IgGl) and AH65 (IgG2a) against human IL-6 were used in DBA/2 mice immunized with purified recombinant IL-6. Among the panel of antibodies obtained, the two most potent in blocking IL-6-dependent growth were selected for further studies. The antibody dissociation constants were determined by Scatchard analysis and were found to be 3.7 pmol/L and 4.8 pmol/L at +4°C for AH64 and AH65, respectively. Anti-IL-6 MoAbs BE8 (IgGl) and BE4 (IgG2b) are commercially available from Innogenics (Besançon, France). These antibodies were previously characterized and successfully used in a therapeutic trial for the treatment of plasmablastic leukemia.

We have measured the clearance of radiolabeled human IL-6 in mice treated with either one or with several murine antibodies of very high affinity for human IL-6. In animals injected with a single MoAb, the mean residence time of IL-6 in plasma is 10 times higher than that in untreated animals, thus leading to a dramatic stabilization of the cytokine. Similar results were obtained with animals treated with two anti-IL-6 antibodies. Surprisingly, in animals treated with a combination of three antibodies binding three distinct epitopes, the clearance of IL-6 was very much enhanced. We showed that this enhancement was mediated by the capture of immune complexes by the receptors for the Fc domain of IgG (Fc receptors), mainly by liver cells. The ternary immune complexes have an enhanced affinity for Fc receptors caused by cyclization and resulting multiple binding, leading to a new route of elimination of these complexes. The use of cocktails of three MoAbs, recognizing three distinct epitopes of a cytokine, provides a new means of antagonizing cytokine activity in vivo.

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analytical grade and purchased from Merck (Darmstadt, Germany). Cell culture reagents were from Flow (Irvine, UK). The buffer designated as PBS-BSA is phosphate-buffered saline (PBS; pH 7.4, containing 20 mmol phosphate, 2 mmol NaH₂PO₄, 0.15 mol NaCl) and 20 g of bovine serum albumin (BSA; Boehringer Mannheim, Mannheim, Germany) per liter. Purified recombinant Escherichia coli-derived human IL-6 was obtained from CLB (Amsterdam, The Netherlands).

**Epitope analysis.** The definition of the epitopes recognized by the different anti-IL-6 MoAbs was performed in IL-6 enzyme-linked immunosassays (ELISAs), using various antibodies as solid phase and tracer. Antibodies were biotinylated with biotin-N-hydroxy-succinimide-1-ester (Boehringer Mannheim) following the manufacturer’s instructions. First, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with a 10 μg/mL solution of anti-IL-6 antibody in PBS and blocked with PBS containing 30 g/L of BSA. The immunosassay was then performed as follows. One hundred microliters of IL-6 solution (1 ng/mL) diluted in PBS-BSA was incubated in coated wells together with 100 μL of biotinylated antibody (1 μg/mL) for 8 to 12 hours at +4°C. The plates were then washed twice with PBS containing 0.5 g/L of Tween 20 and bound biotinylated antibodies were shown with peroxidase-conjugated streptavidin (Jackson, West Grove, PA). The absorbance of the enzyme substrate was determined at 492 nm using an automatic plate reader (SLT, Salzburg, Austria).

**Radioiodination of IL-6.** Radioiodination was performed by the standard chloramine-T method. Briefly, to 2 μg of IL-6 at 0.1 mg/mL in PBS was added 5 μL (0.5 mCi) of ¹²⁵I-NaI (Amersham, Amersham, UK) and 10 μL of a 1 mg/mL solution of chloramine-T in PBS. The reaction mixture was incubated for 15 seconds at 20°C. The reaction was then quenched by the addition of 50 μL of 50 mmol/L glycyltyrosine (Bachem, Bubendorf, Switzerland) and 0.5 mL of PBS-BSA. Separation of proteins and low molecular weight reactants was achieved by gel filtration chromatography with Sephadex G50 (Pharmacia, Uppsala, Sweden). Higher molecular weight fractions containing aggregates were discarded. The material corresponding to the IL-6 peak was diluted in PBS-BSA and stored for up to 1 month at 4°C. The preparation gave a single 22.5-kD radioactive band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and more than 90% of the total radioactive material could be precipitated by trichloroacetic acid (TCA). The specific radioactivity, determined with a commercial ELISA (Immunotech, Marseille, France), was always in the range of 0.6 to 0.8 × 10⁴ cpm/mg. The reactivity of ¹²⁵I-IL-6 with the four anti-IL-6 antibodies was tested in a liquid-phase binding assay using polyethylene-glycol precipitation for separation of bound and free ¹²⁵I-IL-6. More than 75% of labeled IL-6 was always in the range of 0.6 to 0.8 cpn/mol. The reactivity of the tube, containing the cell pellet, was cut out and its radioactivity was determined. Nonspecific binding was determined in the absence of anti-IL-6 MoAb and subtracted from bound radioactivity.

**RESULTS**

**Epitope mapping.** Immunoassays were performed with all possible combinations of two at a time of the following MoAbs: AH64, AH65, BE4, and BE8. The signal pattern observed indicated that the four antibodies belonged to three distinct groups: (1) AH65, (2) BE4, and (3) a third group comprising AH64 and BE8. So as to confirm that these experiments defined three distinct epitopes, we performed immunoassays with a third MoAb present in excess in solution. In immunoassays in which we used solid-phase and tracer antibodies of two different groups, we did not observe any interference by a third antibody from a third group. We confirmed in gel filtration experiments that three different MoAbs, recognizing three distinct epitopes as defined above, bound simultaneously to ¹²⁵I-IL-6 and formed stable ternary
weights. In mice treated with anti-IL-6 antibodies, the clearance of IL-6 (1.60 μh) was almost as rapid as the residence time in plasma, indicating that the molecule was eliminated by renal filtration. In animals treated with one or two anti-IL-6 antibodies, mean residence times (MRTs) in the central compartment were calculated from this new set of data. (We confirmed the pharmacokinetic analysis (Fig 1). The elimination of radiolabeled IL-6 was rapid in untreated animals and in animals treated with a combination of three MoAbs. It was much slower in animals treated with one or two anti-IL-6 antibodies. Mean residence times (MRTs) in the central compartment were calculated from this new set of data. (We found MRTs of 70, 600, and 70 minutes for control mice, mice treated with one antibody, and mice treated with three antibodies, respectively.) In untreated animals, there was no specific localization of IL-6 in any organ. More than 70% of the injected radioactivity was detected in the urine of the animals, indicating that the molecule was eliminated by renal filtration. In animals treated with one or two anti-IL-6 antibodies, we also did not observe accumulation of IL-6 in any organ. In contrast with these results, a striking accumulation of IL-6 was observed in the livers of animals treated with antibodies containing two competitive antibodies, such as BE4 + BE8 + AH64, behaved like combinations of two different antibodies.

**Biodistribution of radiolabeled IL-6.** In biodistribution experiments, the data obtained for the plasma compartment confirmed the pharmacokinetic analysis (Fig 1). The elimination of radiolabeled IL-6 was rapid in untreated animals and in animals treated with a combination of three MoAbs. It was much slower in animals treated with one or two anti-IL-6 antibodies. Mean residence times (MRTs) in the central compartment were calculated from this new set of data. (We found MRTs of 70, 600, and 70 minutes for control mice, mice treated with one antibody, and mice treated with three antibodies, respectively.) In untreated animals, there was no specific localization of IL-6 in any organ. More than 70% of the injected radioactivity was detected in the urine of the animals, indicating that the molecule was eliminated by renal filtration. In animals treated with one or two anti-IL-6 antibodies, we also did not observe accumulation of IL-6 in any organ. In contrast with these results, a striking accumulation of radioactivity occurred in the livers of animals treated with combinations of three antibodies recognizing distinct epitopes of IL-6.

**Gel filtration chromatography of serum samples.** Gel filtration chromatography of serum samples. Gel
filtration chromatography permitted accurate detection of complexes of 450, 350, 180, and 252 kD in plasma samples spiked in vitro with radiolabeled IL-6 and, respectively, no, one, two, or three anti-IL-6 MoAbs able to bind simultaneously to IL-6 (Fig 2). Thus, the technique could be used for further analysis of the molecular form of circulating complexes in treated and untreated mice. In plasma samples from untreated animals injected with radiolabeled IL-6, 85% of the radioactivity was detected in fractions corresponding to molecules of 25 to 30 kD. Conversely, the gel filtration chromatography of plasma samples from animals treated with one anti-IL-6 MoAb indicated that the major form, detectable in the plasma, consisted of monomeric complexes of about 180 kD. Thus, the formation of monomeric immune complexes was responsible for the stabilization of the circulating cytokine. Similarly, complexes of 350 kD were detected in the serum of animals treated with pairs of anti-IL-6 MoAbs. Trimeric complexes of higher molecular weight were detected in plasma samples spiked in vitro with labeled IL-6 and a combination of three antibodies and could be also detected in the plasma of animals treated with the same combination, 5 minutes after IL-6 injection. However, the total amount of radioactive material in this sample was much lower than serum samples from the mice treated with one or two antibodies. One hour after the injection, the peak corresponding to trimeric complexes was no longer detectable. In these plasma samples, the radioactivity was detected in fractions of lower molecular weight corresponding to monomeric and dimeric immune complexes. These experiments confirmed that trimeric immune complexes were eliminated very rapidly. The residual radioactivity in mouse sera corresponded to the monomeric and dimeric complexes formed as a result of the partial reactivity of labeled IL-6 with the three antibodies. The pharmacokinetic analysis led, in fact, to an overestimation of the residence time of IL-6 in animals treated with three antibodies, because the calculation from experimental data took into account the contribution of monomeric and dimeric complexes. The gel filtration experiments suggest that the mean residence time of trimeric complexes in the central compartment was much lower than 84 minutes and was, in fact, close to 5 minutes.

Binding of immune complexes to Fc receptors. First, we analyzed the binding of labeled human IL-6 to the membrane of the murine macrophage P388D1 cells in the presence of murine anti-IL-6 antibodies. Labeled IL-6 was used at a concentration of about 1 nmol/L. There was no significant membrane binding either in the absence of anti-IL-6 MoAb or in the presence of a single antibody, of either IgG1 isotype (AH65, AH65, or BE8), or of IgG2b isotype (BE4). We tested next the effect of different MoAb combinations at the same total antibody concentration. Only combinations comprising three antibodies, recognizing distinct epitopes of IL-6, precipitated labeled IL-6 onto the membrane (Fig 3). Some binding was observed when two antibodies were added. Preincubation of labeled IL-6 with the anti-IL-6 MoAbs before the membrane binding experiment had no effect on the result. Interestingly, the binding of radiolabeled IL-6 to P388D1 cells was completely abrogated in the presence of anti-FcRγ MoAb 2.4G2 at 10 μg/mL, demonstrating that the binding resulted from the interaction of the constant parts of the Ig immune complexes with the membrane Fc receptor and not from a direct interaction of IL-6 with membrane IL-6 receptors. In a second experiment, we determined the dose-response curve of membrane binding. Half-maximal binding of labeled IL-6 occurred at a total concentration
of antibody of 4 ng/mL for the combination (AH64 + BE4 + BE8), i.e., an antibody concentration of approximately 25 pmol/L. Finally, we tested the effect of competing irrelevant mouse Igs the binding of IL-6-containing immune complexes. The membrane binding of trimeric immune complexes was not affected by irrelevant mouse Igs at 1 mg/mL, whereas the binding of dimeric complexes was completely abolished at this concentration of competitor.

DISCUSSION

There are numerous pathologic situations that may benefit from a specific means of antagonizing a cytokine response in vivo, such as, e.g., septic shock and acute inflammatory diseases, cytokine-dependent tumor growth, and reaction to organ transplantation.26,27 Cytokine-binding proteins (CBPs), such as soluble cytokine receptors28 or immunoadhesins comprising fragments thereof,29 have been considered for this purpose, as have anticytokine MoAbs.13,14 Such CBPs might be very useful in specifically countering the deleterious effects of cytokine overproduction associated with a pathologic situation. MoAbs are of special interest, because high-affinity antagonists can be produced readily. The present study was designed to investigate the pharmacokinetics of antibody-based anticytokine therapy.

Cytokines were shown to have a very short residence time in vivo. In rats, IL-6 was shown to be cleared rapidly from the plasma by binding to cell surface receptors (mainly in the liver) and by renal filtration, and then to accumulate transiently in the skin.30,31 The plasma half-life of IL-6 was 20 minutes. We have determined the pharmacokinetic parameters for IL-6 using radiolabeled human IL-6 injected into mice. The mean residence time of IL-6 in the central compartment computed from our experimental data (70 minutes, Table 1) was significantly greater than that reported previously.30 We found a significant contribution of the beta phase in the elimination of IL-6 from the central compartment. We did not detect any transient accumulation of IL-6 in the skin, or in any other organ; most of the radioactivity accumulated in the urine, indicating that renal filtration was the major route of elimination. It should be noted that IL-6 of human origin might behave differently from the autologous murine molecule in its interaction with soluble or membrane receptors.

We had previously shown the accumulation of IL-6 in the form of monomeric immune complexes in patients treated with anti-IL-6 antibody.15 The present data from the mouse model indicate that the mean residence time of IL-6 in animals treated with anti-IL-6 antibody is of the same order
These results are consistent with the earlier paradoxical observation in animal models of septic shock correlated with an increase in cells expressing the highest number of IL-6 receptors. As suggested by several recent reports, Trimeric immune complexes results in the increase of bioavailable IL-6 and may severely limit the therapeutic use of anti-IL-6 antibodies. These results are consistent with the earlier paradoxical observation that the protective effect of anti-IL-6 antibodies in animal models of septic shock correlated with an increase in bioavailable IL-6. Antibodies provide a model for the analysis of the physiology of CBPs such as soluble cytokine receptors. As suggested by several recent reports, antagonist CBPs, including MoAbs, may act in vivo as carriers for their respective ligands.

In contrast with the stabilization observed with one or with two anti-IL-6 antibodies, the clearance of IL-6 increased at least 15-fold in animals treated with a combination of three antibodies that bound simultaneously to IL-6 and formed trimeric immune complexes. What is the mechanism underlying this striking effect? First, all antibodies used in our study had high affinity for IL-6 (Kd, 5 to 10 pmol/L measured at +4°C), leading to very stable complexes with IL-6 (dissociation half-time, >72 hours; F.A.M.-J., unpublished observations). Second, all anti-IL-6 MoAbs were blocking MoAbs, which totally inhibited the binding of IL-6 to its high-affinity receptor at the concentration used in vivo. Thus, significant dissociation of the immune complexes could not occur during the in vivo experiments and the membrane binding of complexed IL-6 was not dependent on antigen-antibody interaction nor on interaction of IL-6 with high-affinity IL-6 receptors. Membrane binding of trimeric immune complexes occurred at low concentrations of three complementary antibodies, but was not significant with only two antibodies able to bind simultaneously to the cytokine. Moreover, the binding of trimeric immune complexes was not affected by high concentrations of irrelevant monomeric antibodies, but was completely abolished by anti-Fc receptor antibody. We may assume then that the high-affinity binding of trimeric immune complexes was caused by multiple binding of the Fc part of the antibody to membrane Fc receptors. The binding equilibrium and the binding kinetics of immune complexes of defined stoichiometry to Fc receptors have been analyzed previously using the model of a multivalent hapten and an antihapten antibody. The results indicated that dimeric and trimeric immune complexes bound preferentially to antigen-presenting cells, even in the presence of high concentrations of competitive irrelevant monomeric antibodies, and were cleared more rapidly from the circulation. In another model, a similar affinity enhancement effect was shown to occur in vitro and in vivo. In animals treated with three antibodies, we observed the accumulation of the radiolabeled IL-6 in the liver immediately after the injection. The liver Kupffer cells were previously reported to be the major site of elimination of soluble immune complexes. Taken together, our results suggest that the enhancement of the clearance of IL-6 in the presence of three antibodies, binding to distinct epitopes, is caused by selective uptake of trimeric complexes by Fc receptors of the cells of the endoreticulum compartment, providing a new route for the elimination of IL-6 in vivo. We observed a striking difference in clearance and in membrane binding between dimeric and trimeric immune complexes that had not been observed in earlier studies with multivalent hapten. The structural basis for these differences remains to be clarified. Complexes of higher order should be tested; however, we failed to identify four MoAbs able to bind simultaneously to IL-6. The respective contribution of the different Fc receptor isotypes, as well as the respective efficiencies of the different antibody isotypes, also remains to be investigated. The production of hybrid human-murine antibodies comprising a constant region of human origin of any isotype is now routine laboratory practice and such hybrids may be considered for the clinical use of anticytokine antibody cocktails.

The modification of the route of elimination of the target molecule may have adverse physiologic effects. FcR cross-linking by trimeric immune complexes may lead to the activation of antigen-presenting cells and to the stimulation of the production of monokines, including IL-6 itself. However, in most cases, the amount of the trimeric complexes to be eliminated is very low compared with the average normal concentration of immune complex in the plasma, which is approximately 10 μg/mL. In myeloma patients, the production of IL-6 was found to average 10 μg/day. Conversely, in a patient suffering from sepsis, the daily IL-6 production was greater than 7 mg and in such extreme cases, the quantity of trimeric complexes may limit the use of MoAbs cocktails.
The present study shows that cytokine-binding proteins have dramatic effects on cytokine pharmacokinetics. These pharmacokinetic phenomena have to be taken into account in the evaluation of potential antagonists, such as soluble cytokine receptors. Specifically, the phenomenon of stabilization of a cytokine by a cytokine-binding molecule might be a strong limitation to the therapeutic efficiency of cytokine-binding antagonists, thus leading to much higher active doses in vivo than those predicted from in vitro pharmacologic studies. The use of a cocktail of three antibodies, binding simultaneously to a cytokine, provides a new means of enhancing the clearance of the target molecule and should help in the design of antibody-based clinical trials by overcoming the problem of the accumulation of monomeric immune complexes. More generally, it should prove possible to manipulate the clearance of a cytokine in vivo and thus to gain information regarding physiologic processes involved in the regulation of the cytokine response.

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