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.

MATERIALS AND METHODS

Antibodies. The MoAbs AH64 (IgG1) and AH65 (IgG1) against human IL-6 were raised 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 (IgG1) and BE4 (IgG2b) are commercially available from Innogenetics (Serezè, France). These antibodies were previously characterized and successfully used in a therapeutic trial for the treatment of plasmablastic leukemia. Anti–IL-6 antibodies at 100 pmol/L completely block IL-6–driven proliferation of both murine cell line B9 or human myeloma cell line XG1. The rat MoAb 2.4G2 (kindly provided by Dr M. Dauenh, Paris, France) reacts with mouse Fc-receptor-γ (FcγR) and blocks the binding of murine IgG to membrane FcγR.

Other reagents. Unless otherwise stated, fine chemicals were 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.
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 NaCl, 0.15 mmol KCl) 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-succinimidyl-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 3 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 peroxysdase-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).

**Radiolabling of IL-6.** Iodination 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 of (0.5 mCi) of 125I-Nal (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 glycyl-glycine (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 BSA. The immunoassay 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 peroxysdase-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).

**Binding assays.** The mouse macrophage cell line P388D1 was maintained in RPMI 1640 supplemented with 10% vol/vol fetal calf serum, 2 mmol/L glutamine, 1% vol/vol nonessential amino acids (100×), 1% vol/vol pyruvate (0.1 mol/L), and 1% vol/vol penicillin-streptomycin (5,000 U/mL). Cells were detached mechanically, washed twice with PBS-BSA, and resuspended in the same buffer at 107 cells/mL. Binding experiments were performed as described by Dower et al:100 μL of the cell suspension, 100 μL of anti–IL-6 antibody solution (either 1, 2, or 3 MoAbs), and 150 μL of radiolabeled IL-6 (106 cpm/mL) in all in PBS-BSA containing 2 mmol/L NaCl, were incubated in a 96-well microtiter plate. After 2 hours at +37°C under agitation, 100 μL of the suspensions was centrifuged for 30 seconds at 10,000g through a polyethylene glycol mixture. The bottom 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.** Immunosassays 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 immunosassays with a third MoAb present in excess in solution. In immunosassays 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

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immune complexes of about 500 kD (see Fig 2A). In the same experiments, we observed that 25% to 30% of 125I-IL-6 was in the form of lower molecular weight complexes corresponding to monomeric and dimeric immune complexes. In fact, the maximal binding of 125I-IL-6 to each of the three MoAbs was 75% to 85% in a liquid-phase assay. This partial reactivity of the labeled IL-6 with each antibody of the combination results in the formation of monomeric/dimeric complexes even in the simultaneous presence of excess amounts of the three MoAbs.

**Pharmacokinetics of radiolabeled IL-6.** The experimental curves were fitted with a biexponential using a 1/y weighting, which gives the best possible fit (Table 1). Fitting with a three-exponential did not lead to better results. The pharmacokinetic parameters were calculated from the coefficients of the biexponential. In all cases, the elimination may be considered as biphasic, which corresponds to a two-phases of elimination; the first and second momentum area under the curve (AUC) and area under the moment curve (AUMC). The elimination of radiolabeled IL-6 was rapid in untreated animals and faster 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 previously to IL-6, because only thiry MoAbs can bind simultaneously to IL-6, because BE8 and AH64 were shown to compete with each other. Conversely, combinations of three 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

| Table 1. Pharmacokinetics of 125I-IL-6 in Mice Treated With Anti-IL-6 Antibodies |
|-----------------|-----------------|-----------------|-----------------|
|                  | Saline          | 1 Ab            | 2 Ab            | 3 Abs           |
| a1 (mL⁻¹)       | 6.95            | 38.3            | 25              | 7.29            |
| a2 (mL⁻¹)       | 30.7            | 31.1            | 17              | 37.7            |
| I1 (min⁻¹)      | 3 × 10⁻³        | 1.08 × 10⁻²     | 8.3 × 10⁻⁴      | 2.0 × 10⁻⁹      |
| I2 (min⁻¹)      | 0.148           | 0.091           | 0.056           | 0.26            |
| Tα (min)        | 231             | 642             | 835             | 345             |
| Tβ (min)        | 4.75            | 13.6            | 12.4            | 2.7             |
| AUC (min · mL⁻¹)| 25.3            | 366             | 304             | 37.7            |
| AUMC (min⁻¹ · mL⁻¹)| 7.7 × 10⁴  | 3.34 × 10⁴     | 2.63 × 10⁴      | 1.80 × 10⁴      |
| C0 (%)          | 38              | 70              | 42              | 45              |
| CL (mL · h⁻¹)   | 2.37            | 0.184           | 0.227           | 1.60            |
| MRTc (min)      | 67              | 524             | 724             | 84              |
| MRTp (min)      | 239             | 387             | 469             | 394             |
| MRTb (min)      | 306             | 911             | 1,223           | 478             |
| Vc (mL)         | 2.65            | 1.43            | 2.38            | 2.23            |
| Vss (mL)        | 12.11           | 2.49            | 3.92            | 12.69           |

Eighteen hours before the experiment, mice were treated with anti-IL-6 antibody AH65 (1 Ab), combinations of anti-IL-6 Abs AH65 + BE8 (2 Abs), AH65 + BE8 + BE4 (3 Abs), or saline. The total dose of antibody injected was 5 µg. Results were expressed as percentage of injected dose in plasma versus time. The data were then analyzed as described in Klein et al. 16 From the fitted parameters of the curve [%ID(t) = a1 · e⁻¹αt + a2 · e⁻¹βt, we calculated the half-lives Tα and Tβ of the two phases of elimination; the first and second momentum area under the curve (AUC) and area under the moment curve (AUMC); the initial dose in plasma (C0); the clearance (CL); the mean residence times in the central compartment (MRTc), in the periphery (MPRTp), and in the body (MRTb); the steady-state distribution volume (Vss); and the plasma volume (Vp).
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 complementary 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.

**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 (AH64, 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 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.
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.\textsuperscript{10,31} 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.\textsuperscript{11} 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.\textsuperscript{17} 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 IL-6. The high-affinity IL-6 receptors in the plasma increases, IL-6 becomes available to those cells expressing the highest number of IL-6 binding sites.

Combinations of three MoAbs containing both AH64 and BE8, which compete for binding to IL-6, are indicated with two stars. The experiment was performed in the absence of anti-IL-6 MoAb. The background of IL-6 binding in the absence of anti-IL-6 MoAb was measured at +4°C, leading to very stable complexes with 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 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 bind 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.

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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 a cytokine in vivo and thus to gain information regarding physiologic processes involved in the regulation of the cytokine response.

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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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