Analysis of the mechanism of action of anti-human interleukin-6 and anti-human interleukin-6 receptor-neutralising monoclonal antibodies
Kalai, M.; Montero-Julian, F.A.; Brakenhoff, J.; Fontaine, V.; de Wit, L.; Wollmer, A.; Brailly, H.; Content, J.; Grotzinger, J.

Published in:
European Journal of Biochemistry

DOI:
10.1111/j.1432-1033.1997.t01-2-00690.x

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Analysis of the mechanism of action of anti-human interleukin-6 and anti-human interleukin-6 receptor-neutralising monoclonal antibodies

Michael KALAI1, Félix A. MONTERO-JULIAN2, Just P. J. BRAKENHOFF3, Véronique FONTAINE1, Lucas DE WIT1, Axel WOLLMER4, Hervé BRAINTY1, Jean CONTENT1 and Joachim GRÖTZINGER4

1 Institut Pasteur de Bruxelles, Département de Virologie, Bruxelles, Belgium
2 Immunotech S. A., Marseille, France
3 Department of Autoimmune Diseases, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands
4 Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany

(Received 25 May/19 August 1997) – EJB 97 0745/3

Anti-human interleukin-6 (human IL-6) and anti-human IL-6 receptor (IL-6R)-neutralising monoclonal antibodies (mAbs) are among the most promising human IL-6-specific inhibitors and have been shown to exert short-term beneficial effects in clinical trials. Simultaneous treatment with different anti-human IL-6 or anti-human IL-6R mAbs was recently suggested to be a potent way to inhibit the action of the cytokine in vivo. Although some of these mAbs are already used, their mechanisms of action and the location of their epitopes on the surface of human IL-6 and human IL-6R are still unknown. Here, we analysed the capacity of several anti-human IL-6 and anti-human IL-6R mAbs to inhibit the interaction between human IL-6, human IL-6R, and human glycoprotein 130 (gp130). We mapped the epitopes of several of these mAbs by studying their binding to human IL-6 and human IL-6R mutant proteins. Our results show that several anti-human IL-6 and anti-human IL-6R-neutralising mAbs block the binding between human IL-6 and human IL-6R, whereas others block the binding to gp130. We provide evidence that some of the latter mAbs inhibit interaction with gp130/1, whereas others interfere with the binding to gp130/2. Our results suggest that residues included in the C'D' loop of human IL-6R interact with gp130/2.

**Keywords:** epitope mapping; interleukin-6; interleukin-6 receptor; glycoprotein 130; monoclonal antibody.

Interleukin-6 (IL-6) is a pleiotropic cytokine mainly involved in the regulation of inflammation, immune responses, and hematopoiesis. IL-6 binds first to a low-affinity α-subunit receptor called IL-6R. The IL-6/IL-6R complex associates with two glycoprotein 130 (gp130) β-receptor chains. The two identical molecules, which are designated here as gp130/1 and gp130/2, form a covalently linked homodimer that transduces the signal [1, 2]. The exact stoichiometry and composition of the high-affinity IL-6 receptor complex at the cell surface membrane are still under debate.

Overexpression of IL-6 has been suggested to participate in the pathogenesis of a variety of clinical disorders, including surgical trauma, rheumatoid arthritis, post-menopausal osteoporosis, chronic autoimmune diseases, Castleman’s disease, multiple myeloma, renal carcinoma and AIDS [1]. To date, the rational design of efficient IL-6 inhibitors was only partially successful. Among the most promising human IL-6 (human IL-6)-specific inhibitors are anti-human IL-6 and anti-human IL-6R-neutralising antibodies. So far, these are the only ones tested in vivo. Anti-human IL-6 mAbs were shown to exert short-term beneficial effects both in animal models of some of the IL-6-related diseases, and in human clinical trials [3–6]. In addition to anti-human IL-6 mAbs, other antibodies raised against human IL-6R were also shown to inhibit the activity of human IL-6 both in vitro and in vivo [7–11]. A few of these anti-human IL-6R mAbs were humanized to prevent the development of human anti-mouse immunoglobulin response in treated patients [12, 13]. Inhibition of human IL-6 and human IL-6R activity by the humanized anti-human IL-6R antibody PM-1 has been shown to be effective for the treatment of patients with rheumatoid arthritis, Castleman’s disease, and multiple myeloma [14].

A major drawback encountered during anti-human IL-6 therapy might prevent the clinical application of such antibodies in the near future. The problem observed in these trials was the accumulation of large amounts of circulating human IL-6 in immune complexes [15, 16]. This stabilised human IL-6 is potentially biologically active and might be released from the immune complex by competing IL-6R [17]. The situation is aggravated in patients that produce large amounts of circulating human IL-6 and soluble human IL-6R [18, 19]. Pharmacokinetics studies in mice injected with radiolabelled human IL-6 have demonstrated that treatment with at least three different anti-human IL-6 mAbs resulted in rapid clearance of the mAbs/human IL-6 complexes, and antibody stabilisation of circulating cytokine was no longer observed [20, 21]. It seems that simultaneous treatment with diff-
different anti-human IL-6 mAbs may therefore be a potent way to inhibit the action of the cytokine in vivo.

These studies suggested that the mAbs included in the cocktails should not compete with each other for the binding of the antigen. The mAbs should be high-affinity human IL-6 binders and should preferentially interfere with the binding of distinct receptor subunits to prevent as much as possible the formation of the signal transducing IL-6 receptor complex. Such a treatment may combine an efficient inhibition of human IL-6 with the long in vivo half-life of free antibodies and the rapid clearance of the polymeric immune complexes.

Use of combinations of anti-human IL-6R mAbs interfering efficiently with interactions between human IL-6, human IL-6R, and gp130 will inhibit human IL-6 action even better and may accelerate the removal of excess concentrations of soluble human IL-6R from circulation. Such a treatment may prove more beneficial for rheumatoid arthritis, Castleman’s disease, and multiple myeloma patients than their injection with PM-1 alone.

Although several anti-human IL-6 and anti-human IL-6R mAbs are already used, their mechanism of action and the location of their epitopes on the structures of the corresponding antigens are still unknown. In this study, we investigated the capacity of several anti-human IL-6 and anti-IL-6R-neutralising mAbs to inhibit the bioactivity of their antigens, studied the mechanism of action of these potent human IL-6 antagonists, identified residues included in their epitopes, and predicted their location on the human IL-6 and human IL-6R structure, respectively.

MATERIALS AND METHODS

Reagents. Unless specified otherwise, the buffer used for dilution in all of the immunoasays was 0.1 M Heps, pH 7.2, 0.15 M NaCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, 2 mM NaN₃, and 20 g/l BSA. The wash buffer was 137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.2 (NaCl/P₄), supplemented with 0.01 % NaN₃, 1% BSA.

Antibodies. Anti-human IL-6 mAbs AH-64, AH-65, and anti-human IL-6R mAb M-91 were obtained from Immunotech and prepared as described [22, 23]. Anti-human IL-6 mAbs B-E4 and B-E8 were kindly provided by Dr J. Wijdenes (Diaclone, Besançon, France) [21, 24]. CLB. IL-6 mAbs nos 8, 12, 14, and 16 and LN1-75-8 (B2) were prepared as described [25, 26].

Anti-human IL-6R mAbs M-37, M-91, M-113, M-164, M182, and M-195 were kindly provided by Dr J. Brochier (Institut National de la Santé et de la Recherche Médicale U291, Montpellier, France) [7]. Anti-human IL-6R mAbs 17.6, 22.1, 32.3, 34.4, 38.8, 48.8, and 50.6 were kindly provided by Dr D. Novick (The Weizmann Institute of Science, Rehovot, Israel) [27]. Anti-human IL-6-R mAb PM-1 and anti human gp130 mAbs AM-64 and AM-66 were a gift from Dr K. Yasukawa (Tosoh Corporation, Kanagawa, Japan) [28].

Wild-type and mutant human IL-6 and soluble human IL-6R proteins. Wild-type human IL-6 and soluble human IL-6R were obtained from Immunotech [22, 23] or prepared as described in [29, 30]. Human IL-6 mutants I32 M, F74E, F78E, R168 M, Q175L, R179E, R179W, M184K, double mutants F74E/F78E, K171N/Q175L, and R179E/R182E and triple mutant R30T/Y31H/I32V were produced as described in [29]. Human IL-6 mutants 2a, double mutant Q159E/T162P, triple mutant 2a/Q159E/T162P, deletion mutant J46, and quadruple mutant DFRD were produced as described in [25, 26, 31, 32]. Production of the soluble human IL-6R mutants was carried out as described in [29, 30].

Radiolabelling of human IL-6 and soluble human IL-6R. Metabolic ³⁵S-labelling of wild-type human IL-6 and soluble human IL-6R was carried out as described in [30]. Human IL-6 was labelled with [³²P] using the chloramine-T method as described previously [21].

Determination of the kinetic parameters of human IL-6 binding by anti-human IL-6 mAbs in equilibrium. Association kinetics. Different concentrations of 1 ml biotinylated mAb diluted in NaCl/P₄, supplemented with 0.2% BSA and 5 mM NaN₃, were added to avidin-coated Eppendorf tubes (Immunotech). Following overnight incubation at 4°C, liquid was discarded and tubes were washed. To each tube was added 0.5 ml ¹²⁵I-human IL-6 at 1.5×10⁵ cpm/ml. After 1, 5, 10, 20, 30, 40, 60, 120, 180, 240, 300, 360, 420, and 1000 min of incubation, liquid was removed, the tubes were washed, and the bound radioactivity was measured using a γ counter.

The affinity constant was calculated using the formula:

\[ K_a = \frac{A_b \times F(t)}{A_b \times F(t) - B_{\text{max}} \times Ab_b \times t} \]

where \( Ab_b \geq T \), \( A_b \) is the initial mAb concentration (equal to the free mAb concentration), \( B \) is the bound human IL-6 concentration, \( B_{\text{max}} \) is the bound human IL-6 maximal concentration (equal to the initial human IL-6 concentration), and \( t \) is time.

Affinity constants. Different concentrations of biotinylated anti-human IL-6 mAb were mixed with an equal amount of human IL-6. Reactions were carried out in a total volume of 1 ml NaCl/P₄, supplemented with 0.2% BSA and 5 mM NaN₃, in Eppendorf tubes. Following overnight incubation at 4°C or 37°C, depending on the experiment, 100 μl Sepharcl avidin (Immunotech) was added to each of the tube. Mixtures were incubated for several h at the corresponding initial temperature. Following centrifugation at 2000 rpm in an Eppendorf centrifuging, the concentration of free human IL-6 in each supernatant was measured by the human IL-6 enzyme immunonassay (EIA-1120 kit (Immunotech) [33]. Affinity constants were calculated using Scatchard plots and the following formula:

\[ K_a = \frac{A_b \times F(t)}{A_b \times F(t) - B_{\text{max}} \times Ab_b \times t} \]

where \( Ab_b \geq T \), \( A_b \) is the initial mAb concentration (equal to the free mAb concentration), \( F \) is the free human IL-6 concentration, and \( T \) is the initial human IL-6 concentration (equal to the total human IL-6 concentration).

IL-6 bioassay. The effect of different concentrations of anti-human IL-6 mAbs on human IL-6 bioactivity was measured using the IL-6-dependent mouse hybridoma cell line B9 as described in [25, 34]. Briefly, cells were first washed and incubated for 12 h in the absence of IL-6. Cells were then cultured for 48 h in flat bottom microtiter plates at a concentration of 104 cells/ml in a total volume of 200 μl/well RPMI 1640 medium supplemented with 5% fetal calf serum, human IL-6, and different concentrations of anti-human IL-6 mAbs. Following incubation, 0.25 μCi/well [³³H]thymidine (Amersham) was added, and cells were incubated for additional 8 h. Cells were then harvested on a Whatman filter and mixed with 2 ml scintillation liquid. The radioactivity incorporated in the cell pellet was determined with a β-counter. A standard range of serial dilutions of human IL-6 was included in every experiment. The specific activity, which is defined as the human IL-6 dose that gave half of the maximal B9 proliferation response, was 3–5 pg/ml.

Immunoprecipitation of ³⁵S-labelled human IL-6 and soluble human IL-6R. Immunoprecipitations were carried out in total volumes of 100 μl of each mAb at a final concentration of 3 μg/ml in Eppendorf tubes and 20% (by vol.) protein A-Sepharose (Pharmacia). 5 μl ³⁵S-labelled human IL-6 or soluble
human IL-6R was mixed with the mAbs in the presence or absence of about tenfold (220 ng/ml) excess of unlabelled human IL-6 or soluble human IL-6R depending on the experiment as indicated in Figs 2 and 6. Following overnight incubation at 4°C on a turning wheel, the Sepharose was washed three times. Immunocomplexes were dissociated in Laemmli buffer, separated by SDS-PAGE, and visualised by autoradiography.

**Antibody cross-reactivity assays.** Competition sandwich ELISA (enzyme-linked immunosorbent assay) was used for the detection of cross-reactivity between the anti-human IL-6 mAbs. mAbs AH-65, AH-64, B-E4, and B-E8 were biotinylated using a protein biotinylation kit according to the manufacturer’s instructions (Boehringer-Mannheim). Distinct microtiter plates were coated overnight with 100 μl/well of a 5 μg/ml solution of each of the anti-human IL-6 mAbs diluted in NaC1/P,. Following incubation, plates were washed and incubated with 50 μl/well serial dilutions of human IL-6 and 50 μl/well 1 μg/ml biotinylated anti-human IL-6 mAb used as the tracer. Following 6 h of incubation at 4°C on an orbital shaker, plates were washed three times and bound biotinylated mAb was detected with alkaline-phosphatase-conjugated Extravidin (Sigma). Following 1 h incubation, plates were washed again and phosphatase activity was measured by adding 150 μl/well p-nitrophenyl phosphate at 1 mg/ml in a buffer containing 1 M diethanolamine, 1 mM MgCl2, adjusted to pH 8.9 with HCl. The color reaction was allowed to develop and the absorbance was measured at 405 nm by an ELISA reader. Assays were carried out in duplicate.

**Mapping the epitopes of anti-human IL-6 mAbs by sandwich ELISA and the use of human IL-6 mutants.** The degree of recognition of human IL-6 mutants /26, Q159E/T162P, 2a, 2a/Q159E/T162P, and DFRD, by the different anti-human IL-6 mAbs was measured by ELISA as described in [25, 26, 35]. Briefly, microtiter plates were coated with 1 μg/ml each of the anti-human IL-6 mAbs in NaC1/P,. After washing, the plates were incubated with different concentrations of wild-type or variant human IL-6. Bound human IL-6 was revealed using biotinylated affinity purified goat anti-human IL-6 and horseradish peroxidase streptavidin (Amersham). Bound peroxidase was detected with 3,3’5,5’-tetramethylbenzidine/H2O2. The potential of the anti-human IL-6 and anti-human IL-6R mAbs to interfere with the binding between human IL-6 and soluble human IL-6R in solution, or the recognition of their complex by AH-65, B-E4, or M-91, was tested as follows. Increasing concentrations of the assayed mAb were added to mixtures containing 85.7 ng/ml human IL-6 and 7.14 ng/ml soluble human IL-6R. Reactions were performed in microtiter plates coated with 5 μg/ml anti-human IL-6 mAb AH-65 [22] (Immunotech) or B-E4 to capture cytokine and with 15 U enzyme/ml anti-human IL-6R mAb M-91-AP (Immunotech) to detect human IL-6/soluble human IL-6R complexes, as described previously [23, 29, 30]. Assays were carried out in duplicate.

**RESULTS**

**Analysis of anti-human IL-6 mAbs.** We tested the cross-reactivity between eight anti-human IL-6 mAbs, using competition sandwich ELISA experiments. The results demonstrated that the mAbs can be divided into at least three distinct epitope groups. Group I includes mAbs AH-64, B-E8, and B-E4, and group II includes mAbs B-E4, 12, and B2, and group III includes mAbs AH-65 and 16. Some competition was also observed between mAbs 12 or B2 from group II and mAb 8 from group I.

We measured the kinetic parameters and the dissociation constants at 4°C and 37°C of several of these mAbs (i.e. AH-64, AH-65, B-E4, and B-E8) and analysed their capacity to inhibit the human IL-6-dependent proliferation of B9 cells (Table 1). The results demonstrated that the mAbs are high-affinity human IL-6 binders and potent inhibitors of its bioactivity. Moreover, the findings suggested that mAbs AH-65 and AH-64 were more potent inhibitors than B-E8, which was used in several preliminary clinical trials [4–6]. B-E4 seemed to be the less potent inhibitor out of the four (Table 1).

Since anti-human IL-6-neutralising antibodies can interfere with the binding of human IL-6 to IL-6R (site 1) or to gp130 (sites 2 and 3) (Fig. 1), we studied the mechanism of inhibition of several of the anti-human IL-6 mAbs in a stepwise approach that followed the different stages of the human IL-6/human IL-6R/gp130 complex assembly in vitro.

**Identification of mAbs that block the binding of human IL-6 to human IL-6R.** Assuming that antibodies which interfere with the binding of human IL-6 to human IL-6R shall not be able to immunoprecipitate the intact human IL-6/soluble human IL-6R complex, we identified first those mAbs that can block the for-

<table>
<thead>
<tr>
<th>mAb</th>
<th>$k_a$</th>
<th>$K_d$ (4°C)</th>
<th>$K_d$ (37°C)</th>
<th>ID$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH-64</td>
<td>7.1 x 10^6</td>
<td>3.7 x 10^-12</td>
<td>1.7 x 10^-12</td>
<td>2.3 x 10^-11</td>
</tr>
<tr>
<td>B-E8</td>
<td>2.9 x 10^6</td>
<td>5.9 x 10^-12</td>
<td>2.2 x 10^-11</td>
<td>1.1 x 10^-11</td>
</tr>
<tr>
<td>B-E4</td>
<td>3.6 x 10^6</td>
<td>1.36 x 10^-12</td>
<td>6.7 x 10^-12</td>
<td>1.2 x 10^-10</td>
</tr>
<tr>
<td>AH-65</td>
<td>5.9 x 10^6</td>
<td>4.8 x 10^-12</td>
<td>4.8 x 10^-12</td>
<td>3.3 x 10^-12</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of anti-human IL-6 mAbs. ID$_{50}$ is the mAb concentration required for half-maximal inhibition of 2 U/ml human IL-6 in the B-9 assay.
Fig. 1. A schematic representation of the three-dimensional model of human IL-6. Receptor binding sites are colored as follows: yellow (human IL-6R, site 1), red (gp130p1, site 2); green (gp130p2, site 3). The locations of a few selected residues or regions are indicated.

**Antih-IL-6 mAbs**

Fig. 2. Identification of antihuman IL-6 mAbs that block the formation of the human IL-6/soluble human IL-6R complex. Immunoprecipitation of 35S-labelled soluble human IL-6R by antihuman IL-6 mAbs in the presence or absence of unlabelled human IL-6. An autoradiograph of an SDS/PAGE analysis of one out of three repeated experiments, which gave very similar results, is presented.

AH-65 and B-E4 inhibit the binding of human IL-6 to gp130. Both AH-65 and B-E4 were able to inhibit the biological activity of human IL-6 (Table 1), however they did not block its binding to human IL-6R (Fig. 2). Therefore, we investigated whether they can interfere with the binding to soluble human gp130. Analysis of the effect of increasing concentrations of B-E4 and AH-65 on the binding of human IL-6 to soluble gp130 in the presence of soluble human IL-6R has demonstrated that both of the mAbs can inhibit the binding to human gp130 (Fig. 3). However, as seen with the biological assay (Table 1), mAb AH-65 is about ten times more efficient than mAb B-E4. Fab preparations of mAb AH-65 gave very similar results suggesting that the effect of the antibody may be direct and not merely steric (Fig. 3).

**Identification of residues included in the epitopes of several anti-human IL-6 mAbs.** The identification of residues included in the epitopes of neutralising antibodies may provide information not only on their mechanism of action, but also yield details concerning the structure and function of the antigen [25, 26, 30, 37]. We used a panel of human IL-6 variants that were produced previously for the identification of residues included in the epitopes of the anti-human IL-6 studied here. In this method, residues included in the epitope of a given mAb are delineated by its failure to recognise the corresponding site-specific single amino acid substitution variants of the antigenic protein.

mAb AH-65 and mAb 16 interact with site 3, whereas B-E4, B2, and B4 interact with site 2. The human IL-6 variant Q159E/T162P and the human/murine-IL-6 chimera-2a were the first to indicate the location of a possible gp130-binding site on the structure of human IL-6 [26, 38]. This combined gp130-binding epitope is located at the beginning of the AB loop and the D helix, and is often referred to as site 3 (Fig. 1).

We compared the degree of recognition of increasing concentrations of these 3 mutants and wild-type human IL-6 by mAbs AH-65, B-E4, and 16. mAb AH-65 bound to the double mutant Q159E/T162P and the human/murine-IL-6 chimera-2a were the first to indicate the location of a possible gp130-binding site on the structure of human IL-6 [26, 38]. This combined gp130-binding epitope is located at the beginning of the AB loop and the D helix, and is often referred to as site 3 (Fig. 1).

We compared the degree of recognition of increasing concentrations of these 3 mutants and wild-type human IL-6 by mAbs AH-65, B-E4, and 16. mAb AH-65 bound to the double mutant Q159E/T162P and the triple mutant 2a/Q159E/T162P. As shown before, mAb 16 hardly reacted with these mutants (Fig. 4A and B) [26, 39].

The results indicated that the epitope recognised by AH-65 is shared with mAb 16 and that both may block the binding of gp130 to site 3. They also suggested that B-E4 might interfere with the binding of gp130 to site 2. This gp130-binding epitope may be disrupted by the substitution of Tyr31 and Val121.
by aspartic acid, Gly35 by phenylalanine, and Ser118 by arginine, resulting in an antagonistic human IL-6 variant called DFRD (Fig. 1) [31].

We produced the DFRD mutant and tested its recognition by B-E4, and several other mAbs. mAbs B-E8, 8, and 16, which were tested as controls, recognised the mutant (data not shown). B-E4, and mAbs included in its epitope group, i.e. B2 and 12 (Table 1), hardly recognised the DFRD mutant (Fig. 4C). Of all of the mAbs studied here, these three mAbs were the only ones to show a significantly decreased binding of the human IL-6 deletion variant A26, in which the first 26 residues starting with the N-terminal of the mature form of the protein were removed (data not shown). These results indicate that B-E4 interferes most probably with gpl30-binding by blocking site 2 and suggest that B2 and 12 may act in a similar way. Residues included in their epitopes are most probably located in the A helix and may also be in the C helix.

Anti-human IL-6 mAbs AH-64, B-E8, and 8 recognise amino acid residues crucial for the binding to human IL-6R. mAbs B-E8, AH-64, and 8 can inhibit the binding between human IL-6 and human IL-6R (Fig. 2B [26]). This suggested that they may recognise residues included in site 1 or located in its proximity (Fig. 1). Therefore, we tested the binding of these mAbs to a panel of human IL-6 mutants in which residues predicted to reside in the human IL-6R-binding epitope were altered [29].

B-E8 did not bind to any of the Arg179 mutants although it recognised all of the other mutants (Fig. 5). AH-64 failed to recognise mutants of Arg179 and showed a significant decrease in the recognition of mutants of Arg168, Phe74, and Phe78 (Fig. 5). mAb 8 failed to recognise mutant F78E, double mutants F74E/F78E and R179E/R182E, and showed a significant decrease in the recognition of mutants of Arg179, indicating that its epitope covers the ends of both the AB loop and the D helix (Fig. 5). These results correlate well with the cross-reactivity observed between these three mAbs and suggest that they have overlapping but distinct epitopes (Table 1, Fig. 1).

Our results suggest that the identification of the epitopes of these mAbs by other techniques such as a peptide scan would have been difficult since they seem to be discontinuous and some of the residues they include are very distant in the primary structure.

Analysis of anti-human IL-6R mAbs. The 14 anti-human IL-6R mAbs studied here are directed against the extracellular domain of human IL-6R and recognise the soluble form of the receptor subunit. All of the antibodies except M-91 and 22.1 are capable of inhibiting the biological activity of human IL-6R in cell culture assays [7, 9, 40–42]. M-91 was suggested to interact with the Ig-like domain of the receptor. Competition was observed between mAbs M-37, M-113, M-164, 32.3, 34.4, 38.8, 48.8, and 50.6, and also between mAbs M-195 and PM-1. No competition was observed between mAbs 17.6, 22.1, and M-182 and any other of the anti-human IL-6R mAbs [7, 40].

Structure/function studies of human IL-6R have suggested that several distinct regions in the molecule are interacting with other molecules in the high-affinity human IL-6 receptor com-
Fig. 5. Differential recognition of site 1 human IL-6 mutants by anti-human IL-6 mAbs B-E8, 8, and AH-64 determined by sandwich ELISA(A). The concentrations of equal amounts of each human IL-6 variant were determined using solid phase coated with mAbs AH-65, B-E8, 8, or B-E4, and biotinylated anti-human IL-6 mAb B-E4 or AH-64 as tracers as indicated. Results are presented as percentages of the values obtained for wild-type (WT) human IL-6. Values are the mean ± SD of at least two independent experiments carried out in duplicate. (ND, not determined).

Fig. 6. Identification of anti-human IL-6R mAbs that block the formation of the human IL-6/soluble human IL-6R complex. Immunoprecipitation of [35S]-labelled human IL-6 by anti-human IL-6R mAbs in the presence or absence of unlabelled soluble human IL-6R. An autoradiograph of an SDS/PAGE analysis of one out of three repeated experiments that gave very similar results is presented.
mAbs was compared with the recognition of the wild-type soluble human IL-6R (Fig. 8). mAbs 17.6 and 22.1 recognised all of the mutants and the wild-type. The recognition of mutant V190G by most of the mAbs was poor suggesting that Val190 is important for the conservation of the structural integrity of the molecule at least locally (Fig. 8).

mAbs M-37, M-113, and M-164 hardly recognised mutants of Ser186, Tyr188, and Val190 (Fig. 8A). The recognition of these mutants by mAbs 50.6, 38.8, 34.4, and 32.3 was less affected (Fig. 8B). Among the latter, the effect on mAb 50.6 was the strongest (Fig. 8B). These results suggest that Ser186 and Tyr188 and perhaps Val190 are included or are in the vicinity of the epitopes interacting with mAbs M-37, M-113, M-164, and probably also 50.6 (Fig. 7). mAbs 32.3, 34.4, 38.8, and 48.8 were much less affected by the substitution of these residues and were also less efficient in inhibiting the binding between human IL-6 and soluble human IL-6R (Figs 8B and 6B). Since some competition exists between the two groups, we suggest that they recognise two distinct but close epitopes.

M-195 and PM-1 showed a slight decrease in the recognition of mutants of Tyr249 and a strong decrease in the recognition of mutants of glutamic acid at positions 296 and 297 (Fig. 8A). mAbs M-182, 34.4, and 38.8 are predicted to interfere with the binding of the human IL-6/human IL-6R complex to gp130p2. Some of the anti-human IL-6R mAbs did not block the binding between human IL-6 and human IL-6R (Fig. 6). Nevertheless, they might inhibit the binding to gp130. This was shown recently to be the case of mAb M-182 [41]. It is not known whether this neutralising mAb interferes with the binding to gp130p1 or gp130p2. We searched for a rapid way to distinguish between the two possibilities.

Two different studies have demonstrated that direct interactions occur between human IL-6R and gp130p2 [43, 44]. So far, no evidence indicating that such interactions also occur between human IL-6R and gp130p2 has been provided. Determining if such contacts exist and the identification of their location may be important for understanding how the high-affinity IL-6 receptor complex is assembled. Since crystal and NMR structures of this complex are unavailable, any additional information concerning contact regions with gp130p2 is of extreme importance for the construction of the corresponding three-dimensional model. Such a model may be useful for the study of the high-affinity receptor complexes of other IL-6-type cytokines.

Discrimination between interference with the binding to gp130p1 or to gp130p2 is not a simple task. As demonstrated above, the anti-human IL-6 mAbs B-E4, and AH-65 interact with residues included in site 2 and site 3 on human IL-6, and interfere with the binding to gp130p1 and gp130p2, respectively. Therefore, we used the anti-site 2 mAb B-E4 to play the role of gp130p1 and the anti-site 3 mAb AH-65 as a substitute for gp130p2. Using these mAbs, we searched for anti-human IL-6R mAbs that would possibly interfere with the binding of the human IL-6/soluble human IL-6R complex to one of the two gp130 subunits.

The formation of human IL-6/soluble human IL-6R complexes and their capture by a solid phase coated with either anti-human IL-6 mAb AH-65 or mAb B-E4, in the presence and absence of various concentrations of each of the different anti-human IL-6R mAbs, was monitored by an alkaline-phosphatase (AP)-conjugated anti-human IL-6R mAb M-91-AP. In such an experimental system, mAbs that interfere with the binding between human IL-6 and human IL-6R will decrease the detection of their complex in both assays in a similar manner. Similar results may be observed if the additional mAbs interfere with the binding of the complex by the tracer mAb M-91-AP. A decrease in the binding of the human IL-6/human IL-6R com-
A

<table>
<thead>
<tr>
<th>mAb</th>
<th>17.6</th>
<th>22.1</th>
<th>M-37</th>
<th>M-113</th>
<th>M-164</th>
<th>M-182</th>
<th>PM-1</th>
<th>M-195</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding (% of WT)</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

**shIL-6R**

**shIL-6R**

**Fig. 8. Identification of residues included in the epitopes of anti-human IL-6R mAbs by sandwich ELISA and soluble human IL-6R mutants.**

Amino acid substitutions are indicated by the single-letter code. Results are presented as percentages of the values obtained with the wild-type (WT) soluble human IL-6R. Values are the mean of 2–4 independent experiments carried out in duplicate.

The results obtained with mAbs M-37, M-164, M-195, PM-1, 32.3, and 48.8 correlated well with expectations based on the immunoprecipitation experiments (Fig. 6). All of these mAbs inhibited the formation of the human IL-6/human IL-6R complex in a concentration-dependent manner in both of the experimental systems. The results obtained for M-195 are shown as an example (Fig. 9).

Anti-human gp130 mAb AM-66 which was used as a negative control did not affect the recognition or the formation of the complex (data not shown). Several other mAbs, which were able to immunoprecipitate efficiently radiolabelled human IL-6 in the presence of soluble human IL-6R (Fig. 6) nevertheless affected significantly the recognition of the human IL-6/soluble human IL-6R complex in one or both of the experimental systems. mAb 17.6 showed inhibitory effects even at low concentrations (data not shown). This interference was independent of the anti-human IL-6 used in the assay and was dose dependent only at low concentrations. These results might imply that mAb 17.6 interferes with the binding of human IL-6/soluble human IL-6R complex to the tracer M-91-AP. The effect of mAb 22.1 was minor in both cases though somewhat stronger when the phase mAb was AH-65 (data not shown).

The results obtained for mAbs M-182, 34.4, and 48.8 correlated better to those obtained previously (Fig. 6) when the solid phase was coated with mAb B-E4 (Fig. 9). When the solid phase was coated with AH-65 these monoclonal antibodies were inhibitory. The degree of inhibition varied between high for mAbs 38.8 and 34.4, and low for mAb M-182 (Fig. 9). It seems therefore, that the binding of these anti-human IL-6R mAbs to the human IL-6/soluble IL-6R complex interferes with its capture by the anti-human IL-6 mAb AH-65 probably because of steric hindrance.

These results suggest that mAbs 34.4, 38.8, M-182, and 22.1 may also interfere with the binding of the human IL-6/soluble human IL-6R complex to gp130β2. Analysis of their effect on the binding to gp130 indicated that mAbs 34.4, and 38.8 are more efficient inhibitors of the binding between the human IL-6/soluble human IL-6R complex and soluble gp130 than mAb M-182 (Fig. 10).
Fig. 9. Interference of anti-human IL-6R mAbs with the formation of the human IL-6/soluble human IL-6R complex or with its detection by anti-human IL-6 mAbs AH-65 (■) or B-E4 (□) and anti-human IL-6R mAb M-91.

Fig. 10. Dose-dependent inhibition of the binding of the human IL-6/soluble human IL-6R complex to soluble human gp130 by anti-human IL-6R mAbs 34.4, 38.8, and M-182.

**DISCUSSION**

**Anti-human IL-6 mAbs.** The present study and previous results show that anti-human IL-6 mAbs AH-64, B-E8, 8, 12, B2, B-E4, AH-65, and 16 are all potent inhibitors of human IL-6 bioactivity. These antibodies can be divided into at least three distinct epitope groups.

mAbs AH-64, B-E8, and 8 interact with residues included in site 1 (Fig. 1) and block the binding of human IL-6 to human IL-6R. mAbs 12, B2, and B-E4 interact with residues included in site 2 (Fig. 1) and probably block the binding to gp130β1. mAbs AH-65, and 16 interact with residues included in site 3 (Fig. 1) and block the binding to gp130β2. These results support all previous findings concerning the location of the different receptor binding sites on the human IL-6 structure and the residues they include [26, 29, 38, 39, 46].

The data we present may help in choosing which mAbs would be most beneficial in the treatment of human IL-6-related disease and in which combinations. It is suggested that the antibodies used in such a treatment will include representatives of each of the three epitope groups. To inhibit efficiently the bioactivity of human IL-6 such mAb cocktails could include the following: mAbs AH-64 or 8 to block site 1, mAbs B-E4 or B2 to block site 2 and mAbs AH-65 or 16 to block site 3.

**Anti-human IL-6R mAbs.** We compared many anti-human IL-6R mAbs to facilitate the choice of mAb combinations to use in anti-human IL-6R clinical treatment. Our findings and previous results demonstrate that the antibodies can be divided into at least six distinct epitope groups.

Our results show that the anti-human IL-6R mAbs M-37, M-113, M164, M-195, and PM-1 are strong inhibitors of the binding of human IL-6 to human IL-6R, whereas mAbs 32.3, 48.8, and 50.6 affect the reaction to a lesser degree. They suggest that residues Ser186, Tyr188, and Val190 are located in the epitope recognised by anti-human IL-6R mAbs M-37, M-113, M-164, and 50.6 or in its vicinity, and that residues Glu296, Glu297, and Tyr249 may interact with mAbs PM-1, and M-195 (Fig. 7). The results in this study support further our previous suggestion that these residues are exposed on the surface of the human IL-6R molecule and that Tyr188, Tyr249, Glu296, Glu297, and maybe also Val190 are directly interacting with human IL-6 [29, 30]. Moreover they suggest that the EF, B'C', and FG' loops of human IL-6R are immunogenic.

The results concerning mAbs M-182, 17.6, 22.1, 32.3, 34.4, 38.8, and 48 are less direct. We have shown here that mAbs M-182, 17.6, 22.1, 34.4, and 38.8 do not inhibit the binding between human IL-6 and human IL-6R. mAb 17.6 showed a slight competition with M-91 for the binding of the human IL-6/human IL-6R complex. This effect was not observed with human IL-6R alone. It is therefore suggested that mAb 17.6 recognises an epitope distinct from the one interacting with mAb M-91, although it is probably close enough to allow for steric hindrance.

We showed that mAbs 34.4, 38.8, and M-182 inhibit the binding of the human IL-6/human IL-6R complex to gp130. Since they interfered also with the binding of this complex to anti-human IL-6 mAb AH-65, which was shown here to recognise residues included in the human IL-6 site 3 (Fig. 4), we suggest that like AH-65, these mAbs interfere with the binding to gp130β2.

Interestingly, the region recognised by mAb 34.4 was identified recently by a peptide scan. The sequence common to the four peptides that it interacts with corresponds to residues Tyr257−Thr266 in the CD' loop of domain II of human IL-6 (Fig. 7, green circle). Moreover, the peptide that reacts best with mAb 34.4 was shown to inhibit the activity of human IL-6 in different biological assays and was suggested to interfere with the binding to gp130β2.

In our three-dimensional model of the human IL-6/human IL-6R/human gp130β1 complex [47], the corresponding region is exposed on the surface of the human IL-6R with an orientation similar to that of site 3 in human IL-6 (Figs 1 and 7). Interestingly, the alteration of several residues included in the CD' loop was shown to reduce significantly the bioactivity of the corresponding soluble human IL-6R variants (i.e.double mutant E260A/R261G and triple mutant R261G/S262A/K263G), but did not affect their human IL-6-binding capacity [43]. Taken together, these results suggest that residues included in the CD' loop of human IL-6R are interacting with gp130β2.
human IL-6R mutagenesis studies could verify this prediction. This information may help the construction of a three-dimensional model of the human IL-6 high-affinity receptor complex.

In analogy with the anti-human IL-6 mAbs, combining antibodies such as M-37 or M-164 with M-195 or PM-1, and 34.4, 38.8, or M-182 in the treatment of IL-6-related disease may also result in an efficient inhibition of the human IL-6 activity and rapid clearance of soluble human IL-6R.

We thank Dr D. Novick, Dr J. Brochier, Dr J. Wijdenes, and Dr K. Yasukawa for anti-human IL-6R, anti-human IL-6, and anti-human gp130 mAbs which were used in this study. We thank Dr G. Muller-Neven for critical reading. This work was supported by grants from the CGER (Caisse Générale d’Epargne et de Retraite) Fonds de Recherche Cancérologique and the Fund for Medical Scientific Research (Belgium).

REFERENCES

700

Kalai et al. (Eur. J. Biochem. 249)


