Dimerization and activation of the common transducing chain (gp130) of the cytokines of the IL-6 family by mAb

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Abstract

The objective of our research was to study the mechanisms of activation of mAb against the gp130 transducer chain common to the IL-6 cytokine family. It has been found that among the 56 anti-gp130 available worldwide, none was able to activate the growth of IL-6-dependent myeloma cell lines. When certain of them were associated in pairs they allowed the cells to grow; alone, they were inhibitory. The same activation was also obtained by cross-linking certain anti-gp130 mAb on the cell membrane with a goat anti-mouse Ig antiserum. A bispecific mAb was prepared by the somatic fusion of two hybridomas secreting two mAb whose association was able to activate gp130 signaling; the bispecific mAb was inactive. The activating mAb were able to support long-term proliferation of the IL-6-dependent myeloma cell lines, which indicates that they are potential valuable growth factors of tumor cells and hematopoietic stem cells. When they were injected into SCID mice, they allowed human IL-6-dependent myeloma cell lines to grow, develop tumors and metastasize. By studying the functional epitopes of the cell membrane gp130 receptors, it was shown that the activating mAb induced gp130 dimerization and STAT3 activation, as did IL-6.

Introduction

The transmembrane gp130 chain is used for transducing the signals of the cytokines of the IL-6 family [IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1)] after it has bound the complex formed by the cytokines and their specific receptor (1). This molecule is present on almost all mammalian cells and has been shown to be a vital receptor since the deletion of its gene in mice is lethal (2). The cytokines which are able to activate gp130 are involved in many inflammatory and tumoral diseases (1). Inhibition of their deleterious activity may have therapeutic effects (1). We and others have produced anti-gp130 mAb to inhibit gp130 signaling (3,4). The mAb appear to be very interesting inhibitors since, depending on their specificity, they are able to selectively inhibit activation induced either by IL-6, IL-11, CNTF, LIF or OM (3,5). This shows that particular epitopes of the extracellular domain of the gp130 receptor are specifically involved in binding each of the cytokine–receptor complexes.

Some of the anti-gp130 mAb show an agonist activity and are able to sustain gp130-dependent cell proliferation (4,6). These mAb appear to be interesting, original tools and reagents. They are potent gp130 activators which can be used to elucidate gp130 signaling. They can also be used as growth factors of tumoral cells which are needed to develop specific immunotherapy and growth factors of hematopoietic stem cells. Less expensive to produce than recombinant cytokines, stimulating anti-gp130 mAb are well adapted to industrial production. For this, a precise knowledge of their mode of action and the conditions of activation is necessary. We demonstrate here that two mAb directed against two different epitopes and paired together...
are necessary to achieve gp130 activation, and that anti-gp130-induced activation results from gp130 dimerization.

**Methods**

**mAb and reagents**

Thirty-seven mAb, all IgG1, were obtained by somatic hybridization of spleen cells from BALB/c mice immunized with recombinant gp130 and SP2-0 myeloma cells, which recognized 10 main groups of epitopes called A–J (A1-4, B1-3, B5, C1-10, D1-5, E1-3, F1-3, G1-4, H1, I1-2 and J1) (3). mAb of groups B and E were shown to inhibit the binding of IL-6–IL-6 receptor (IL-6R) complexes to gp130; those of groups A, I and J inhibited any activation induced by the IL-6 family of cytokines; mAb C2 recognized an epitope with an unknown function (3). Anti-gp130 mAb B-S12, prepared by J. Wijdenes (Diaclone, Besançon, France), was obtained from the panel of mAb provided by the Vth International Workshop on Human Leukocyte Differentiation Antigens, held in Osaka, Japan, 1996. Anti-IL-6 mAb B-E8 was kindly given by J. Wijdenes. Anti-IL-6R mAb M195 (neutralizing IL-6 binding) and M91 (not neutralizing) (7), and mAb CD138, MI15 which recognized human Syndecan-1 antigen specifically expressed by human Ig, were kindly given by J. Wijdenes. Anti-IL-6 mAb B-E8 was kindly given by J. Wijdenes. Anti-IL-6R mAb M195 (neutralizing IL-6 binding) and M91 (not neutralizing) (7), and mAb CD138, MI15 which recognized human Syndecan-1 antigen specifically expressed by human plasma cells (8), had been prepared in the laboratory. Purified IL-6 was generously provided by Sandoz Forschungsinstitut (Vienna, Austria). The IL-6 antagonist Sant5 (9) was kindly given by G. Ciliberto. Genistein and staurosporin were purchased from Sigma (St Louis, MO).

**Preparation of bispecific anti-gp130 mAb**

Hybridomas secreting mAb B1 and I2 were made by HGPR and TK deficient by culturing them in the presence of 20 µg/ml Azaguanine or BrdU (Sigma) respectively. They were hybridized with PEG as previously described (7), and cultured in RPMI 1640 medium containing hypoxanthine, aminopterin and thymidine (HAT; Sigma). Four clones were selected which secreted mAb against B1 and I2 epitopes. Bispecific mAb were purified by HPLC on DEAE 5PW (Beckman Instruments, Gagny, France) from one of the clones called H22.1.

**Affinity columns for gp130 absorption**

CNBr-activated Sepharose beads (Sigma) were coated with anti-gp130 mAb C1 or I1 according to the manufacturer’s procedure. Then 300 µl of gel coupled with 0.6 mg of either mAb was used to absorb 48 µg of gp130 and then 10 µg of B-S12, followed by extensive washing. The two columns were then washed with glycine buffer (0.1 M, pH 3) and the eluted material dialyzed against PBS. The eluted material from the column coated with mAb C1 was called I-eluted B-S12 (because the C epitopes were masked) and that eluted from the other column was called C-eluted B-S12.

**ELISA**

**Epitope analysis was performed using a sandwich ELISA. Plates (Immunon I; Nunc, Roskilde, Denmark) were coated overnight at 4°C with one of the anti-gp130 mAb diluted in PBS, washed 6 times with PBS containing 0.05% Tween 20 (Sigma) and saturated with 1% BSA in PBS for 1 h at room temperature. After incubation with recombinant gp130 diluted in PBS/BSA (1% BSA in PBS containing 0.05% Tween 20), followed by six washings, incubation with a biotinylated anti-gp130, six more washings, incubation with peroxidase-labeled streptavidin (Immunotech, Marseilles, France) and eight final washings, the reactivity was determined by the intensity of the enzymatic reaction to the substrate o-phenylene diamine measured by absorbency at 492 nm after the addition of half a volume of 2 N H2SO4.

Murine and human Ig, soluble human IL-6R, and gp130 were measured in mouse plasma by the sandwich ELISA already described (3,7) using anti-murine Ig and anti-human Ig antisera (Jackson Immunoresearch, Westgrove, PA) and peroxidase-labeled anti-murine Ig and anti-human Ig antisera (Jackson Immunoresearch), M182 and biotinylated M91, and A1 and biotinylated D2 mAb respectively.

**Cell lines**

XG-1 and XG-2 myeloma cell lines were prepared by our group (10). Their growth was strictly dependent on the presence of exogenous IL-6. BAF cells transfected with gp130 (BAF190), gp80 + gp130 (BAF80/130) or gp190 + gp130 (BAF190/130) human genes were prepared by one of us (B. K.) and J. F. Moreau. XG-1 and XG-2 myeloma cell lines and BAF80/130 were maintained in culture in RPMI 1640 medium containing 10% FCS in the presence of IL-6, BAF190 in the presence of IL-6–IL-6R complexes and BAF130/190 in the presence of LIF.

**Cell activation**

In order to assay activation, cells were washed 4 times with RPMI 1640 medium, incubated for 4 h at 37°C in RPMI 1640 medium containing 10% FCS but no stimulator and washed twice again. Washed XG cells were resuspended at 10^5/ml in RPMI 1640 medium containing 20% FCS. Then 50 µl of this suspension was distributed in 96-well flat-bottomed microtitration plates to which 50 µl of mAb (alone or in association) diluted in RPMI 1640 medium was added. In each plate a positive control of proliferation (50 µl of medium with IL-6 but without anti-gp130 mAb) and a negative control without IL-6 were done in triplicate. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO2 until the growth in the best growing cultures, estimated by examination under a microscope, appeared to reach an optimum, generally at day 5. Each well received 0.5 µCi of [3H]thymidine (25 Ci/mole; Amersham, Arlington Heights, IL) for 12 h before the cells were collected with a cell harvester (Skatron, Lier, Norway). Radioactivity was counted in a liquid scintillation β counter (TriCarb 1900 CA; Packard, Meriden, CT) and expressed in c.p.m.

**Western blotting**

With a view to studying gp130-induced signaling, XG-2 cells were washed twice and cultured (2x10^5 cells/ml) for 12 h at 37°C in IL-6-deprived RPMI 1640 medium containing 1% of FCS, then agonist anti-gp130 mAb (50 µg/ml each) or IL-6 (100 ng/ml) were added for 1, 5, 10, 30 and 60 min. Cells were rapidly washed and immediately lysed at 4°C in 10 mM Tris–HCl, pH 7.05, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 1% Triton X-100, 5 mM ZnCl2, 100 mM Na3VO4, 1 mM Triton, 20 mM β-glycerophosphate, 20 mM
nitsuphenol phosphate, 2.5 mg/ml aprotinin, 2.5 mg/ml leupeptin, 0.5 mM PMSF, 0.5 mM benzamidin, 5 mg/ml pepstatin and 50 nM okadaic acid. After preclearing at 14,000 g, soluble supernatant was redisssolved in 10% SDS–PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked for 1 h at room temperature in 138 mM NaCl, 3 mM KCl, 25 mM Tris–HCl (pH 7.4), 0.1% Tween 20 (TBS-T). 5% BSA, then incubated for 1 h at room temperature with primary antibody (phospho-specific STAT3 at 1:1000 dilution and anti-STAT3 at 1:2000 dilution in 1% BSA TBS-T). Rabbit phospho-specific STAT3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and mouse anti-STAT3 from Transduction Laboratories (Lexington, KY). Binding of the primary antibodies was visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Jackson Immunoresearch) peroxidase-conjugated antisera (at 1:10 000 dilution in TBS-T) and by an enhanced chemiluminescence detection system. The membranes were twice stripped with 100 mM glycine, pH 2.2, 0.1% NP-40 and 1% SDS for 30 min.

Flow cytometry

Cells were washed with PBS containing 1% BSA and 0.02% sodium azide (PBS/BSA), incubated with various concentrations of IL-6 or anti-gp130 mAb diluted in PBS/BSA, then washed twice and incubated with the appropriate dilution of anti-IL-6R or anti-gp130 mAb for 1 h. They were washed twice again and incubated with a FITC-labeled goat anti mouse Ig antiserum (Immunotech, Marseilles, France), washed twice finally and then analyzed by cell flow cytometry (FACScan; Becton Dickinson Mountain View, CA) using the CellQuest software. The cells were also stained using biotinylated IL6 or anti-gp130 mAb revealed with phycoerythrin-labeled streptavidin (Immunotech).

Animal facilities

SCID/bg/bg (CB-17/lcrHsc-scid-bg) mice were purchased from Harlan (Cannat, France) and bred in our animal house. Mice at 5–6 weeks old were tested for the presence of circulating Ig. Leaky mice (<10%) were discarded. Washed XG-2PA cells (5×10⁷) were injected i.p. Then 50 µg of mAb B1 and I2 (25 µg each) were injected i.v. the same day followed by i.v. injections of 25 µg (12.5 µg of each) every 3 weeks. The mice were examined carefully every week. Between 100 and 200 µl of blood was taken on heparin every fortnight from the orbital sinus. The plasma was aliquoted and stored at −20 °C until needed. In more recent experiments, the mice were irradiated 24 h before cell infusion with a total dose of 2 Gy, delivered at 1 Gy per min, by a CsCl blood irradiator.

Results

Two different anti-gp130 mAb are necessary to activate gp130 signaling

As participants in the VIth International Workshop on Human Leukocyte Differentiation Antigens (11), we assayed the 56 different anti-gp130 mAb submitted to the Workshop and found only one (B-S12) able to activate IL-6-dependent myeloma cell lines. In a previous paper (6) we suggested that BS12 could be a mixture of two different mAb because it was the only mAb of the panel to react against two different epitopes of the gp130 molecule [called C and I in our nomenclature (3)], whereas all the others recognized only one. This led us to use mixtures of two different mAb and we found a few which were able to activate IL-6-dependent myeloma cell lines. Since it seemed important to us to be able to answer the question of whether or not two mAb were necessary to activate gp130, we absorbed aliquots of B-S12 selectively on C or I epitopes (gp130 bound by a type I mAb coated to Sepharose beads exposes C epitopes, whereas gp130 bound by a C mAb exposes I epitopes) and eluted the absorbed mAb as shown in Fig. 1. The C-eluted mAb reacted with epitope C but not with I and vice versa (Fig. 1). This clearly showed that B-S12 was a mixture of mAb C and I. Among the mAb that were produced in our laboratory, we found that a mixture of anti-gp130 mAb C5 + I1 was as potent as B-S12 to activate IL-6-dependent myeloma line: B1 + I2, B2 + I1, B1 + F1, A3 + I1 and B2 + C5. We have since

Fig. 1. Selective absorption of B-S12 mAb on C and I gp130 epitopes. (Upper part) gp130 was captured by mAb C or I covalently linked to Sepharose beads and 10 µg aliquots of B-S12 mAb were passed on it. After PBS washing, the absorbed material was eluted at acid pH and dialyzed against PBS. (Lower part) The anti-gp130 specificity of the eluted material was studied by using the cross-blocking ELISA already described (3,7) The wells were first coated with an anti-gp130 mAb (as indicated), washed, incubated with recombinant gp130, washed, incubated with a biotinylated anti-gp130 (as indicated) and washed again. The binding of biotinylated mAb was revealed using peroxidase-conjugated streptavidin and o-phenylene diamine. Due to the absence of repetitive epitopes on the gp130 molecule (3), absence of binding of a biotinylated mAb indicated the specificity of the captured mAb.
Table 1. Stimulation of various cell lines by anti-gp130 mAb

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<tr>
<th>Cell line</th>
<th>Stimulating mAb</th>
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<tbody>
<tr>
<td></td>
<td>B1 + I1 or B2 + I2</td>
</tr>
<tr>
<td>XG-1</td>
<td>–</td>
</tr>
<tr>
<td>XG-2</td>
<td>–</td>
</tr>
<tr>
<td>BAF80/130</td>
<td>–</td>
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<tr>
<td>BAF130</td>
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<tr>
<td>BAF130/190</td>
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Cells were washed, incubated in new medium for 4 h, washed again and cultured for 5 days in the presence of anti-gp130 mAb as indicated or with IL-6 taken as a positive control. [3H]Thymidine was incorporated for another 18 h.

**Key:** ++, >75% of the IL-6 stimulation taken as a maximum; +, >25% and <75% of the IL-6 stimulation taken as a maximum; ±, <25% of the IL-6 stimulation taken as a maximum; –, no stimulation.

Prepared two new mAb of type B (B3 and B5) which react in the same way as B1. For the present study, we selected the pair of mAb B1 + I2, which is one of the most potent activating associations; on the other hand, the pair of mAb B1 + I1 was inactive (although I1 and I2 are able to cross-block each other) and served as a negative control.

Since activation could possibly be due to IL-6 synthesis and the autocrine use of it, neutralizing anti-IL-6 (B-E8) and anti-IL-6R (M195) mAb were added to XG-2 cells activated by IL-6 or B1 + I2. No diminution of the growth induced by the mAb was ever seen, whereas that induced by IL-6 was completely inhibited (Fig. 2). Certain anti-gp130 mAb, for instance the A2 (Fig. 2), were able to interact and inhibit the proliferation induced by agonist anti-gp130 mAb.

**Preparation and activity of bispecific anti-gp130 mAb**

As indicated in Methods, we hybridized B1 and I2 hybridomas and selected 1 clone (H22.1) secreting one bispecific mAb recognizing both B1 and I2 epitopes. The Protein A-purified IgG was found to be as potent as B1 + I2 to activate XG-2. However, the production of this clone is a mixture of the 10 predicted chain associations, including mAb B1 and I2. The HPLC-purified bispecific mAb was unable to activate XG-2 cells although it reacted against both B and I epitopes (data not shown).

**Activation of different lines**

To find out whether the stimulating mAb were able to activate, equally well, any cell line expressing gp130, we tested the six active mixtures of mAb on another IL-6-dependent myeloma cell line, XG-1, and found very similar results, except that the pair B2 + C5 was hardly active on XG-1 (Table 1). When transfected BAF cells were used different results were obtained. BAF80/130 cells were activated by both B1 + I2 and B2 + I1, while BAF130 was activated by B1 + I2 only, whereas BAF190/130 were never activated at all (Table 1). When flow cytometry was carried out, the various activating anti-gp130 mAb recognized gp130 on the surface of the five cell lines equally well.

**Goat anti-mouse Ig cross-linking**

In another series of experiments we attempted to stimulate cell growth by cross-linking only one anti-gp130 mAb with a goat anti-mouse antiserum. Only cross-linked mAb of type B or E were able to achieve a significant activation of XG-2 cells (Table 2).

**Long-term stimulation**

In order to discover whether the stimulating mAb could sustain long-term cell proliferation, XG-2 cells were cultured, in parallel, either with IL-6 as usual or with mAb B1 + I2 without IL-6 (subline called XG-2PA). Cumulative cell production showed that the stimulating mAb were as potent as IL-6 (Fig. 3A) except that there was a 1–2 day latency at the beginning of the culture. In the absence of an extrinsic factor, the XG-2PA cells died; they remained IL-6-dependent although they tended to grow better in the presence of B1 + I2, whereas the XG-2 cells tended to grow better in the presence of IL-6 (Fig. 3B). These slight differences appeared to be highly reproducible.

In order to see whether XG-2 and its subline XG-2PA might use different intracytoplasmic signaling, the two lines were cultured with two inhibitors of phosphorylation: genistein and staurosporin. Whether they were stimulated by IL-6 or B1 +
Table 2. Stimulation of various cell lines by anti-gp130 mAb cross-linked with a goat anti-mouse Ig antiserum

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Stimulating mAb</th>
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<tbody>
<tr>
<td></td>
<td>B1, B2, I1, I2, A3, C5 or F1</td>
</tr>
<tr>
<td></td>
<td>B1 + GaMlg</td>
</tr>
<tr>
<td></td>
<td>B2 + GaMlg</td>
</tr>
<tr>
<td></td>
<td>I1, I2, A3, C5 or F1 + GaMlg</td>
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<tr>
<td></td>
<td>E1</td>
</tr>
<tr>
<td></td>
<td>E1 + GaMlg</td>
</tr>
<tr>
<td>XG-2</td>
<td>–</td>
</tr>
<tr>
<td>BAF80/130</td>
<td>–</td>
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<tr>
<td>BAF130/190</td>
<td>–</td>
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</tbody>
</table>

Key: ++, >75% of the IL-6 stimulation taken as a maximum; +, >25% and <75% of the IL-6 stimulation taken as a maximum; ±, <25% of the IL-6 stimulation taken as a maximum; –, no stimulation.

GaM, goat anti-mouse.

Fig. 3. Comparative stimulation of XG-2 and XG-2PA cells by IL-6 and mAb B1 + I2. (A) XG-2 cells were cultured at 100,000 cells/ml in the presence of IL-6 (1 ng/ml) or of the mixture of mAb B1 + I2 (2.5 µg/ml of each). Cells were counted every 4–5 days and put back in culture at 100,000 cells/ml, for a total duration of almost 8 weeks (exactly 52 days). (B) Thymidine incorporation by XG-2 and XG-2PA cells cultured for 5 days in the presence of IL-6 (1 ng/ml), mAb B1 + I2 (2.5 µg/ml of each) or PBS.

I2, the two lines were inhibited equally well by both genistein (Fig. 4A) and staurosporine (Fig. 4B).

Signal transduction induced by agonist anti-gp130 mAb
Both the B-S12 mAb and the B1 + I2 mixture were found to induce STAT3 phosphorylation in IL-6-deprived myeloma XG2 cells (Fig. 5). The kinetics of phosphorylation was slightly delayed as compared to that of IL-6. We failed to immunoprecipitate gp130 on XG-2 cells (or other XG cell lines), presumably due to a too low gp130 concentration as already described (12).

Fig. 4. Dose-dependent inhibition of XG-2 and XG-2PA cell growth by staurosporine and genistein. XG-2 and XG-2PA cells were cultured for 6 days in the presence of IL-6 (1 ng/ml) or mAb B1 + I2 (2.5 µg/ml each) as indicated and various concentrations of staurosporine (A; ng/ml) or genistein (B; µg/ml) were added at the beginning of the culture. Proliferation was measured by [3H]thymidine incorporation for 18 h and expressed in c.p.m.

gp130 dimerization
To find out how the ligands interact with their receptors on the cell membrane, the expression of various gp130 epitopes was studied by flow cytofluorometry with the corresponding mAb. Five were used: (i) M195, directed against an IL-6R epitope involved in IL-6 binding (7), (ii) B1, directed against
Stimulating anti-gp13 mAb

Fig. 5. Tyrosine phosphorylation of STAT3 induced in XG-2 cells stimulated by agonist anti-gp130 mAb or IL-6. XG-2 cells were washed twice and starved of IL-6 for 12 h in RPMI 1640 and 1% of FCS at 37°C. They were stimulated with 100 µg of a pair of agonist anti-gp130 mAb (50 µg each) or IL-6 (100 ng/ml) for 1–60 min. The lysate of the unstimulated or stimulated cells (1 × 10^6 cells per lane) was subjected to SDS–PAGE electrophoresis and immunoblotted with a phospho-specific STAT3 antibody (lane A). The blot was stripped and reprobed with an anti-STAT3 antibody (lane B). Mol. wt markers on the right are in kDa.

Fig. 6. Schematic view of the functional epitopes involved in IL-6–IL-6R–gp130 interaction on the cell membrane. mAb M195 inhibits the binding of IL-6 to IL-6R, mAb B1 inhibits the binding of the IL-6–IL-6R complex to gp130 and mAb A1 inhibits the cytokine-induced gp130 activation. mAb M91 and C2 are not inhibitory.

Table 3. Ligand-induced gp130 dimerization on the membrane of XG-2 cells as shown by cytofluorometry analysis.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Ligand</th>
<th>PBS</th>
<th>IL-6</th>
<th>Sant 5</th>
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<tbody>
<tr>
<td>M195</td>
<td>+</td>
<td>− or +</td>
<td>− or +</td>
<td></td>
</tr>
<tr>
<td>M91</td>
<td>+</td>
<td>− or +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>+</td>
<td>− or +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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Washed XG-2 cells were incubated for 30 min at 0°C with IL-6, Sant 5 or PBS as a negative control. Epitope analysis of IL-6R and gp130 molecules was done using anti-IL-6R mAb M91 and M195, and anti-gp130 mAb A1, B1 and C2 respectively.

Incubation with B1 + I2 did not induce the rapid and selective diminution of expression of the A1 epitope.

Growth of XG-2PA cells in SCID mice injected with stimulating anti-gp13 mAb

In a final series of experiments we studied whether the activating mAb B1 + I2 were able to support the growth of XG-2PA cells in SCID/bg mice. A preliminary experiment had shown that the half life of mAb B1 + I2 was 13 days when injected i.v. into these mice. A mixture of mAb B1 + I2 was injected into six mice, 50 µg/mouse at day 0, then 25 µg/mouse every 3 weeks, which made it possible to maintain a concentration of circulating mAb between 5 and 30 µg/ml. At day 0, 5 × 10^7 XG-2PA cells were injected i.p. After 15 days, no XG-2PA cells could be seen in the circulation when CD138,MI15 mAb were used to detect them. The persistence and growth of XG-2PA cells was studied by measuring the concentration of released human Ig in the plasma of the mice (Fig. 8). This concentration rose slightly 2 weeks after injection, then declined slowly. In five mice, it rose again rapidly around weeks 8–16, with no clinical sign of a tumor. A few weeks...
Stimulating anti-gp13 mAb 1887

Fig. 7. Expression of A1, B1 and C2 gp130 epitopes on XG-2 cells. Histograms were established by fluorescence analysis on a FACScan of living cells gated by forward- and side-scatter properties. (Upper part) Washed XG-2 cells were incubated with 25 µg/ml of anti-gp130 mAb A1, B1 or C2, then with a FITC-labeled goat anti-mouse Ig antiserum (gray histograms) and compared with control mouse IgG1 (white histograms). (Lower part) Same experiment as above, except that washed XG-2 cells were incubated with 100 ng/ml IL-6 for 30 min in ice in the presence of 0.02% NaN3 before being stained with anti-gp130 mAb (white histograms) and compared with cells not incubated with IL-6 (gray histograms).

Table 4. Ligand-induced gp130 dimerization on the membrane of XG-2 cells as shown by cytofluorometry analysis

<table>
<thead>
<tr>
<th>mAb</th>
<th>Ligand</th>
<th>PBS</th>
<th>B1 + I2</th>
<th>B1 + I1</th>
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<tbody>
<tr>
<td>M195</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>A1</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
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<td>C2</td>
<td>+</td>
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Washed XG-2 cells were incubated for 1 min at 37°C with anti-gp130 mAb B1 + I2 or B1 + I1 as indicated, and PBS as a negative control, and then washed. Epitope analysis of IL-6R and gp130 molecules was done using anti-IL-6R mAb M195 and anti-gp130 mAb A1 and C2 respectively.

later an abdominal tumor appeared. Three of the mice were killed; they showed a solid tumor on the peritoneal membrane. The cells of the tumor stained strongly with CD138,MI15 mAb and they were put in culture where they died unless cultured with IL-6 or mAb B1 + I2. At the time of autopsy, a consistent number of cells (~50%) which stained with mAb CD138,MI15 were found in the blood, the spleen and the bone marrow of the mice. Untreated mice never developed tumors, neither did mice treated with mAb B1 or I2 alone or with XG-2PA cells associated with the non-stimulating mixture B1 + I1.

Since a wide variability in the efficiency and timing of the

Fig. 8. Tumor formation by injection of XG-2PA cells in SCID mice treated with mAb B1 + I2. SCID/bg mice treated with mAb B1 + I2 as indicated in the text were injected i.p. with 5×10^7 XG-2PA cells. Blood was taken every fortnight to measure mouse (not shown) and human Ig. No clinical signs of tumors were seen when the human Ig started to rise. Mouse 4 died 12 weeks after cell injection. Mice 2, 3 and 5 were killed 12, 25 and 18 weeks after cell injection respectively. At autopsy, a solid tumor was found on the peritoneal membrane. Cells of the tumor, blood, spleen and bone marrow were studied for the presence of the specific plasma cell antigen detected by mAb CD138,MI15.
..., mice were subjected to 2 Gy irradiation 24 h before cell infusion. Eighty-eight percent (21 of 24) of the animals treated with B1 + I2 were able to inhibit the formation of tumors induced by B1 + I2 (Fig. 9).

**Discussion**

Gp130 activation by cytokines of the IL-6 family implies that the receptor is dimerized either with itself or with the gp190 LIF-R (1). One can expect certain anti-gp130 mAb to achieve such homodimerization and to activate gp130 signaling. For this we tested the 56 mAb submitted to the VIth International Workshop on Human Leukocyte Differentiation Antigens which was held in Osaka, Japan, in 1996. We found only one mAb (B-S12) able to activate the growth of IL-6-dependent myeloma cell lines (6,11), which confirmed results already published (4,13). Since B-S12 appeared to be the only one of the 56 mAb to possess two epitope specificities (6,11), it was absorbed on and eluted from gp130, which showed that it was a mixture of two different anti-gp130 mAb. Therefore, none of the 56 (or 57) available anti-gp130 mAb was able to activate gp130 signaling alone. Such mAb should exist since one mAb directed against the erythropoietin receptor (from 44 tested) was able to do so as shown in a recent publication (14). However, the probability of getting one mAb able to trigger gp130 dimerization alone appears to be very low.

By pairing mAb that had been produced in our laboratory, we were able to reproduce an association similar to that of B-S12 and we found a few other pairs able to activate the growth of IL-6-dependent myeloma cell lines. The mAb involved in activation all belong to groups of epitopes directly involved in the formation of complexes between gp130 and its ligands. Used alone they are inhibitors (B inhibits IL-6–IL-6R binding to gp130 (3), A and I inhibit gp130 dimerization, C inhibits OMP and LIF activation, and F inhibits CNTF activation (3–5)]. mAb of group E which inhibit IL-6–IL-6R binding to gp130, in the same way as those of group B (3), were inactive in any association but were able to trigger gp130 activation when aggregated with goat anti-mouse Ig. It seems logical that mAb targeting of the domains involved in the formation of membrane complexes can lead to activation, but why two different mAb are needed still remains unexplained. It could simply be a way of reaching a sufficient affinity necessary to achieve gp130 dimerization. However, how can one explain that the associations of B1 + I2 and B2 + I1 are active, whereas those of B1 + I1 and B2 + I2 are not, when B1 and B2 (and I1 and I2) cross-block each other and possess a similar affinity for gp130 (3)? These activating associations show a certain selectivity since not all of them activate the growth of every cell line bearing gp130. It could be that the presence, on the cell membrane, of other receptor chains surrounding gp130 plays a certain role. mAb B and E, which are able to inhibit the binding of IL-6–IL-6R to gp130 (3), can activate XG-1 and XG-2 cells when cross-linked by a goat anti mouse Ig antiserum. Under the same conditions, mAb B activates BAF80/130 cells but not BAF130 or BAF130/190. The presence of IL-6R might favor gp130 activation by these mAb; in other terms, the mAb can replace IL-6 to some extent. However, the B1 + I2 mixture did not lead to IL-6R association with gp130 since the M195 epitope was still free on the XG-2 cells. Moreover, neutralizing anti-IL-6 and anti-IL-6R mAb were unable to inhibit anti-gp130-induced proliferation.

Upon addition of IL-6 to IL-6-dependent cells, phenotyping of gp80 and gp130 epitopes easily dissects IL-6–IL-6R–gp130 interactions; in particular, the disappearance of the A1 epitope is a sign of gp130 dimerization. We show here that mAb B1 + I2 were able to trigger gp130 dimerization, as did IL-6. The immediate diminution of the A1 epitope was followed by a relatively rapid disappearance of the gp130 chains, which is not likely to play an important role in gp130 activation since the inactive pair of mAb B1 + I1 caused the same gp130 redistribution. The dimerization leads to an intracytoplasmic signaling which may be similar to that induced by IL-6. At least it was inhibited similarly by non-specific protein kinase inhibitors. Furthermore, agonist anti-gp130 mAb were found to induce tyrosine phosphorylation of STAT3 similarly to IL-6, in agreement with previous data (4). Precise comparison between the inducing action of cytokines and of mAb of the phosphorylation of gp130 and the multiple cytosolic proteins on different cell lines would be necessary to go further. mAb directed against the erythropoietin receptor were reported to be less efficient in supporting cellular growth than erythropoietin (14), which, according to the authors, could mean that mAb-induced dimerization is less efficient than that induced by the natural ligand. This is not so obvious in our case where some pairs of activating mAb were nearly as potent as IL-6.
From a practical point of view, it is particularly interesting that activating mAb were able to support long-term cellular growth. One possible application is the culture of tumor cells necessary to achieve tumor cell-based specific immunotherapy, such as tumor antigen vaccination. These mAb appear to be interesting growth factors and are a valuable alternative to the use of the corresponding cytokines. Anti-gp130 mAb have been shown to be co-activators of hematopoietic stem cells (4,13). As it is hoped that hematopoietic stem cells amplified in vitro will, in the future, replace those obtained from bone marrow or from blood after their mobilization, these activating mAb could become very valuable tools. When injected into SCID mice, due to their relatively long life (t₁/₂ = 13 days), the activating mAb turned these mice into transgenic-like animals for genes of cytokines of the human IL-6 family. Moreover, since they do not react against murine gp130, the mAb do not show any toxicity as compared with human IL-6 which binds to the murine IL-6R and causes deleterious reactions (15). In SCID/bg mice treated with activating mAb, IL-6-dependent human myeloma cells were able to grow, develop tumors and even metastasize. This makes an easy and relatively inexpensive model of human cancerization which is invaluable for studying new targets of therapy as well as the pharmacology of new drugs.

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Abbreviations

CT-1 cardiophrin 1
CNTF ciliary neurotrophic factor
IL-6R IL-6 receptor
LIF leukemia inhibitory factor
OM oncostain M
SCID severe combined immune deficiency
STAT signal transdencer and activator of transcription

References