In Vitro Killing of Neuroblastoma Cells by Neutrophils Derived From Granulocyte Colony-Stimulating Factor–Treated Cancer Patients Using an Anti-Disialoganglioside/Anti-FcyRI Bispecific Antibody

By Jean Michon, Sandrine Moutel, Jacques Barbet, Jean-Loup Romet-Lemonne, Yashwant M. Deo, Wolf H. Fridman, and Jean-Luc Teillaud

Neutrophils isolated from cancer patients treated with granulocyte colony-stimulating factor (G-CSF) express high levels of FcyRI. They exhibited an efficient killing of GD2 neuroblastoma in the presence of an anti-disialoganglioside (GD2) mouse monoclonal antibody (MoAb; 7A4, IgG3κ). However, this cytotoxicity was totally blocked by human monomeric IgG. In contrast, a bispecific antibody (7A4 bis 22/MDX-260), prepared by chemically linking an F(ab’) fragment of 7A4 with an F(ab’) fragment of an anti-FcyRI MoAb, 22, which binds outside the Fc binding domain, triggered antibody-dependent cell cytotoxicity, even when neutrophils were preincubated with human monomeric IgG. F(ab’), 22 MoAb abrogated the MDX-260 killing without affecting that of 7A4. The 3G8 MoAb, directed against the FcyRIII binding site, did not inhibit the cytotoxicity induced by either antibody. Thus, these results indicate that G-CSF–activated neutrophils exert their cytotoxic effect against neuroblastoma cells through FcyRI and not FcyRII, and that the saturation of the high affinity FcyRI by monomeric IgG can be overcome by the use of bispecific antibodies binding epitopes outside the IgG FcyRI binding site. A combined administration of such bispecific antibodies and G-CSF may be, therefore, an efficient therapeutic approach to trigger tumor lysis by cytotoxic neutrophils in vivo.

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proach for specific immunotherapy using in vivo stimulated neutrophils as effector cells and engineered MoAbs.

**MATERIALS AND METHODS**

*Isolation of neutrophils.* Neutrophils were isolated from 2 mL of peripheral blood of 24 adult patients with non-Hodgkin’s lymphoma or breast or ovarian carcinoma or of eight children with non-Hodgkin’s lymphoma, Ewing’s tumor, rhabdomyosarcoma, retinoblastoma, neuroblastoma, or osteosarcoma. All the adults patients were treated with recombinant human G-CSF (r-metHuG-CSF, filgrastim; Amgen, Neuilly-sur-Seine, France) for more than 10 days during recovery from various chemotherapy regimens. The juvenile donors were incubated overnight with 200 IU/mL of G-CSF and morphprep (Nycomed, Oslo, Norway) density gradient was used to purify neutrophils. Neutrophils from 24 adult patients with non-Hodgkin’s lymphoma, Ewing’s tumor, rhabdomyosarcoma, retinoblastoma, neuroblastoma, or osteosarcoma. All the adults patients were treated with filgrastim for 5 days (three cases) to collect peripheral blood hematopoietic progenitors. or received filgrastim during recovery from various chemotherapy regimens. The juvenile patients were treated with filgrastim for 5 days (three cases) to collect peripheral blood hematopoietic progenitors, or received filgrastim after chemotherapy (five cases). All the patients tested were included in clinical studies approved by ethics committees. Signed informed consent was obtained from each adult patient or from parents of juvenile patients before their entering these clinical trials. Polymorphprepp (Nynomed, Oslo, Norway) density gradient was used to purify neutrophils. Neutrophils (3 × 10^7/mL) from healthy adult donors were incubated overnight with 200 IU/mL of G-CSF and 200 IU/mL of interferon-γ (IFNγ; Roussel-Uclaf, Romainville, France) in RPMI 1640 culture medium before use. Neutrophils from cancer patients were immediately used after isolation. Neutrophil preparations were at least 95% pure as shown by May-Grünwald-Giemsa staining.

*Cell lines.* The Go2^− LAN 1, IMR 32, and ACN 75 neuroblastoma cell lines were provided by Dr D. Beck (CHUV, Lausanne, Switzerland). The melanoma cell lines HT 144 and 902 B were provided by Drs E. Tartour (Institut Curie, Paris, France) and M. Herlyn (The Wistar Institute, Philadelphia, PA), respectively. The Go2^− ovarian carcinoma cell line SW 626 was provided by Dr P.-A. de Jaco (Clinica Ostetrica e Ginecologica II, Bologna University, Bologna, Italy). All tumor cell lines were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (FCS; Seromed), 1% L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Experiments were performed on trypsinized cells (Trypsin-EDTA; GIBCO, Paisley, UK) subsequently washed in 10% FCS-containing culture media.

*Antibodies.* The hybridoma B cell lines 7A4 (mouse IgG3, κ, anti-Go2) and PS1.1 (IgG3, κ, anti-phosphorylcholine) were provided by Drs D. Beck and N. Gross (CHUV) and by Prof M.D. Scharff (Albert Einstein College of Medicine, New York, NY), respectively. MoAbs were purified from culture supernatants by affinity chromatography onto protein G-Sepharose (Pharmacia, Uppsala, Sweden), and their purity was checked by sodium dodecyl sulfate (SDS)-gel electrophoresis and enzyme-linked immunosorbent assay (ELISA).

Alkaline phosphatase-labeled anti-human IgM goat antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). Fluorescein isothiocyanate-labeled F(ab′)2, IgG goat-antimouse Ig (GAM-FITC; Southern Biotechnology) was used in indirect immunofluorescence assays. FITC-labeled mouse IgG1 was obtained from Immunotech (Marseille, France). The following MoAbs were supplied by Medarex (Annandale, NJ): IV3 F(ab′)2 (IgG2b, κ), directed against FcyRII; 3G8 F(ab′)2 (IgG1, κ), directed against FcyRIII (CD16); and 22 F(ab′)2 and FITC-coupled 22 MoAb (IgG1, κ) directed against FcγRI. Polyvalent human immunoglobulins (HulG; 50 mg/mL) were obtained from the Centre National de Transfusion Sanguine (Les Ulis, France). Heat-aggregation of Hu-IgG (2 mg/mL in RPMI 1640 culture medium) was performed by a 20-minute incubation at 63°C.

*Bispecific anti-Go2/anti-FcγRI antibody.* The 7A4 bis 22 BsAb [F(ab′)2 × F(ab′)2], designated MDX-260, was prepared as previously described by Glennie et al.17 at Immunotech (Marseille, France) using 7A4 and 22 MoAb F(ab′)2 fragments and bis-maleimide as crosslinking reagent (Fig 1). It was purified by preparative gel filtration on Superdex 200 (Pharmacia). Two separate batches of this BsAb were prepared. The purity of these preparations was over 90% as determined by analytical Superdex 200 chromatography. A major component with an apparent molecular mass of 110 kD, the expected molecular mass of a bispecific (F(ab′)2 × F(ab′)2) antibody, was detected by SDS-7.5% polyacrylamide gel electrophoresis (PAGE) in nonreducing conditions, as were some additional molecules with apparent molecular masses of 85, 50, and 25 kD. The BsAb stock solutions were stored at 4°C.

*FcγRI-μ fusion protein.* A cell culture supernatant of transfected COS 7 cells supplied by Medarex containing a fusion protein exhibiting the extracellular domains of FcγRI and the constant region of human IgM (FcγRI-μ fusion protein), was used to detect the MDX-260 BsAb binding to both Go2 and FcγRI molecules by ELISA.

*ELISA.* LAN 1 cells were harvested from culture flasks after trypsinization, washed, and resuspended in phosphate-buffered saline (PBS) at 10×10^6/mL. Cells were then sonicated, and the cell extract was stored at −20°C until further use. ELISA microplates (Maxisorp; Nunc, Rockslide, Denmark) were coated with 50 μL of cell extract per well (corresponding to a concentration of 5 × 10^6 sonicated cells per well) for 2 hours at room temperature. After washing, the plates were saturated with 1% low-fat milk–PBS overnight at 4°C. Antibody samples (MDX-260, 7A4, or PS1.1, 100 μg/mL, 50 μL per well) were twofold diluted in 50 μL of PBS containing 1% bovine serum albumin (BSA; Fraction V, Sigma Chemical Co, St Louis, MO). After a 2-hour incubation at 20°C, plates were washed, and FcγRI-μ COS 7 supernatant (1:4 diluted in PBS-1% BSA) was added (50 μL per well). After a 2-hour incubation at 20°C, plates were washed and incubated for 90 minutes at 37°C with alkaline phosphatase-labeled antihuman IgM (1:250 in PBS-1% BSA) goat antibodies before the addition of substrate (p-nitro-phenyl phosphate disodium). Absorbance was read at 405 nm using an ELISA microplate reader (Tietertek Multiscan; Labsystems, Les Ulis, France).

*Immunofluorescence assays.* Direct immunofluorescence assays were performed by incubating 10^6 cells on ice for 30 minutes with FITC-labeled antibodies diluted in 50 μL of PBS-1% BSA. Indirect immunofluorescence assays were performed by incubating 10^6 cells for 30 minutes on ice with the first antibody (10 μg/mL). Cells were then washed twice in RPMI 1640 containing 5% FCS and incubated for another 30-minute period with GAM-FITC diluted in PBS-1% BSA. After two washes in PBS, cells were fixed with PBS-1% formaldehyde, and 5,000 cells per experiment were analyzed with a FACSScan cytometer (Becton-Dickinson, Pont de Clai, France), using the Lysis program.

*Complement-dependent cytotoxicity assay.* One hundred micro-

Fig 1. Schematic diagram of the MDX-260 BsAb. Q represents N-ethyl succinimidyl; R, O-phenylendisuccinimidyl.
liters of LAN 1 cells (2×10^6/mL in RPMI 1640) was incubated with 100 μL of either 7A4 MoAb (4 μg/mL) or MDX-260 BsAb (8 μg/mL) in RPMI 1640 for 30 minutes on ice, washed with 5 mL RPMI 1640, and incubated twice with 200 μL of low-cytotoxic 1:4 diluted serum from young rabbit (IFA-CREDO, Marcy-l'Etoile, France) at 37°C for 45 minutes. Cytotoxicity was evaluated using the trypan blue exclusion assay.

**ADCC assay.** Target cells (3×10^6 to 10×10^6) were incubated for 1 hour at 37°C with 7,400 μCi of ³¹Cr (37 mCi/mL; New England Nuclear DuPont, Bad Homburg, Germany). After two washes in culture medium, 5×10^6 [³¹Cr]-labeled cells were plated in each well (V-bottomed, 96-well tissue culture plates; Nunc). Assays were performed in triplicates in a final volume of 200 μL per well. Plated target cells were then incubated with various antibody preparations (7A4, P51.1, MDX-260) or culture medium at 37°C for 30 minutes before the addition of effector cells. The [³¹Cr] minimum release was determined by counting the supernatant of 5×10^4 target cells incubated with culture medium only (spontaneous release). The maximum release was estimated by incubating 5×10^6 [³¹Cr]-labeled target cells with 2 N HCl. Effector cells were plated at different effector:target (E:T) cell ratios ranging from 100:1 to 12.5:1. In some experiments, effector cells were first plated and incubated with various amounts of monomeric or heat-aggregated HulgG or with F(ab')2 anti-FcyR MoAbs (20 μg/mL) for 15 minutes at room temperature before adding antibodies and target cells.

Plates were centrifuged for 20 seconds at 80g, incubated for 4 hours at 37°C in a 5% CO₂ humidified atmosphere, and centrifuged again at 425g for 2 minutes. Supernatant (100 μL from each well) was counted using a MultiGamma counter (Pharmacia Wallac). The mean value of triplicates was used to calculate the percentage of specific [³¹Cr] release as:

\[
\text{Specific Release} = \frac{\text{Sample Release (cpm) - Spontaneous Release (cpm)}}{\text{Maximum Release (cpm) - Spontaneous Release (cpm)}} \times 100
\]

**RESULTS**

**Binding of MDX-260 BsAb to FcyRI⁺ neutrophils or Gd2⁺ neuroblastoma cells.** Table 1 shows that neutrophils isolated from G-CSF–treated cancer patients express high levels of FcyRI, as compared with healthy volunteers. In G-CSF–treated patients, expression of FcyRI is significantly increased, whereas FcyRIII is markedly reduced and FcyRII is slightly decreased.

Both the 22-FITC MoAb and the MDX-260 BsAb bind to FcyRI⁺ neutrophils, while both the 7A4 MoAb and the MDX-260 BsAb bind to Gd2⁺ LAN 1 cells (Fig 2). In addition, the 7A4 MoAb and the MDX-260 BsAb also bind to two other Gd2⁺-expressing neuroblastoma (ACN 75 and IMR 32) and one Gd2⁺-expressing melanoma (HT 144) cell lines. No binding to the Gd2⁺ SW 626 ovarian carcinoma and the Gd2⁺ 902 B melanoma cell lines was observed (data not shown).

**Table 1. Expression of FcyRI (CD64), FcyRII (CD32), and FcyRIII (CD16) on Neutrophils Isolated From Cancer Patients Undergoing G-CSF Treatment**

<table>
<thead>
<tr>
<th>G-CSF Patients (n = 8)</th>
<th>Controls (n = 4)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcyRI</td>
<td>93±12</td>
<td>11±4</td>
</tr>
<tr>
<td>FcyRII</td>
<td>120±39</td>
<td>230±39</td>
</tr>
<tr>
<td>FcyRIII</td>
<td>137±89</td>
<td>2,754±39</td>
</tr>
</tbody>
</table>

Data are expressed as mean fluorescence channel ± SD in arbitrary units. Controls were healthy volunteers. P values were determined by Mann-Whitney U test.
Cross-linking of GD2 to FcγRI by MDX-260 BsAb. The ability of MDX-260 BsAb to bind to both GD2 and FcγRI was investigated by an indirect ELISA using the FcγRI-µ fusion protein. Both the MDX-260 BsAb batches were tested. Figure 3 shows that the MDX-260 BsAb simultaneously binds to both GD2 and FcγRI. Similarly, the anti-GD2 parental 7A4 MoAb, a mouse IgG3 that binds human FcγRI, could also be detected in this dual binding assay. The half-maximum concentration for the whole 7A4 MoAb was about 10-fold less than the F(ab')2 X F(ab')2 BsAb. This is an expected result, as the 7A4 MoAb is bivalent with respect to GD2 binding, whereas MDX-260 BsAb is monovalent.

Lack of complement-dependent cytotoxicity by MDX-260 BsAb. The complement-dependent cytotoxicity of 7A4 MoAb or MDX-260 BsAb on LAN 1 cells was evaluated. MDX-260 BsAb did not mediate complement-dependent cytotoxicity, whereas 85% to 90% cytotoxicity was obtained with 7A4 MoAb (data not shown). Again, this is an expected result due to the presence of the Fc region in the 7A4 mouse IgG3 MoAb.

Neutrophil-mediated ADCC by 7A4 MoAb and MDX-260 BsAb. MDX-260 was then examined for its ability to trigger ADCC of LAN 1 cells by neutrophils from G-CSF-treated cancer patients. [51Cr]-labeled LAN 1 cells were lysed in the presence of either 7A4 or MDX-260 antibodies, whereas no cytotoxicity was observed when only neutrophils or neutrophils in the presence of an irrelevant IgG3 MoAb (P51.1) were tested (Fig 4). A similar cytotoxicity was obtained at all E:T ratios with MDX-260 BsAb as compared with 7A4 MoAb when these antibodies were used at 1 µg/mL. Studies of 10 donors indicated that both antibodies used at this concentration induced about 60% [51Cr]-specific release at a 100:1 (E:T) ratio (7A4: 65.1% ± 12.9% v 7A4 bis 22: 58.5% ± 13.1%; mean ± SEM). However, dose-dependent experiments indicated that the 7A4 MoAb may be more efficient in lysing LAN 1 cells at lower concentrations (≤0.15 µg/mL; data not shown). More than 60% of [51Cr]-specific release was still obtained when using 0.15 µg/mL of 7A4 MoAb, whereas the same concentration of MDX-260 BsAb provoked a 35% to 40% toxicity. The other GD2+ cell lines (ACN 75, IMR 32, and HT 144) were also efficiently lysed with the 7A4 MoAb and the MDX-260 BsAb, whereas the GD2- SW 626 ovarian carcinoma cell line and the GD2- 902 B melanoma cell line were not (Table 2).

When neutrophils from normal healthy individuals were tested as effector cells, no cytotoxicity was observed with either 7A4 MoAb or MDX-260 BsAb. However, these neutrophils could lyse LAN 1 cells with both 7A4 and MDX-260 antibodies after 24-hour cultivation in the presence of G-CSF and IFNγ (data not shown).

Inhibition of cytotoxicity by HuIgG. High-affinity FcγRI binds to monomeric HuIgG (IgG1 and IgG3) and is, therefore, likely to be saturated in vivo by plasma-circulating IgG, inducing a so-called antibody blockade. In addition, in vitro, heat-aggregated HuIgG blocks the binding site of all three FcγRs (FcγRI, II, and III), whereas monomeric IgG only blocks FcγRI. All these receptors are expressed on neutrophils derived from G-CSF-treated cancer patients (Table 1). Therefore, the ability of monomeric or heat-aggregated HuIgGs to block neutrophil-mediated ADCC against LAN 1 cells was investigated. We first analyzed whether monomeric HuIgGs inhibited the 7A4 MoAb- or the MDX-260 BsAb-mediated cytotoxicity by neutrophils derived from G-CSF-treated cancer patients. The 7A4 MoAb-mediated cytotoxicity was inhibited in a dose-dependent manner by monomeric HuIgG, whereas the MDX-260 BsAb-mediated cytotoxicity was not inhibited in a dose-dependent manner by monomeric HuIgG.
cytotoxicity was not affected (Fig 5). The incubation of neutrophils with heat-aggregated HuIgG also blocked the 7A4 MoAb-mediated cell cytotoxicity in a dose-dependent manner, without inhibiting the cytotoxicity mediated by the MDX-260 BsAb (Fig 5). Thus, MDX-260 MoAb mediates ADCC of LAN1 cells via FcγRI, even in the presence of human monomeric IgG. In contrast, the addition of 22 F(ab′)2 MoAb to neutrophils inhibited the MDX-260 BsAb-mediated ADCC, but did not affect the 7A4 MoAb-mediated ADCC (Fig 6). Similarly, the addition of IV.3 or 3G8 F(ab′)2 MoAbs, which interfere with FcγRII and FcγRIII IgG binding sites, respectively, did not provoke any inhibition of cell cytotoxicity (Fig 6), thus demonstrating that neither FcγRII nor FcγRIII is involved in 7A4- or MDX-260-mediated ADCC by in vivo G-CSF–activated neutrophils.

**DISCUSSION**

In vivo, G-CSF induces a rapid increase of circulating neutrophil counts and is widely used to reduce neutropenia-related morbidity in cancer patients receiving chemotherapy. Recent data have indicated that neutrophils from G-CSF-treated patients show an increased expression of the high-affinity FcγRI, which is a trigger molecule for ADCC. We show here, using an anti-GD2/anti-FcγRI BsAb (MDX-260), that these neutrophils can efficiently lyse GD2+ cell lines in vitro through the engagement of FcγRI. The lower killing efficiency observed at low concentrations of MDX-260 BsAb, as compared with the parental 7A4 MoAb, is likely caused by the monovalence of BsAb.

The crucial role of FcγRI in the antitumor activity of neutrophils from G-CSF–treated patients has been suggested by the lack of inhibition of the ADCC activity by MoAbs directed against low-affinity FcγRs. In the present study, F(ab′)2 MoAbs that interfere with the IgG-binding site of FcγRII and FcγRIII had no inhibitory effect on ADCC. However, the engagement of FcγRII expressed on G-CSF-activated neutrophils to mediate ADCC cannot be excluded, as this low-affinity receptor is unable to bind mouse IgG3 subclass (to which 7A4 MoAb belongs) as well as MDX-260 BsAb. It has been reported that the IV.3 MoAb, directed against the IgG-binding site of FcγRII (CD32), which is likely to be responsible for the MoAb-mediated cell cytotoxicity exerted by freshly isolated neutrophils, had no effect on ADCC mediated by neutrophils derived from G-CSF–treated individuals. Thus, FcγRI is probably the only FcγR able to mediate ADCC by activated neutrophils. In addition, the data reported here confirm that FcγRI, and not FcγRIII, is an important trigger molecule for the ADCC exerted by neutrophils derived from G-CSF–treated cancer patients. An anti-FcγRI MoAb, 197, that blocks IgG binding to FcγRI, has been previously reported to inhibit up to 50% of the cytotoxicity of G-CSF-activated neutrophils. Furthermore, a BsAb (MDX-210) that binds to an epitope of FcγRI located outside the IgG binding site and to an epitope of the c-erbB-2 protein product of the HER-2/neu protooncogene could promote ADCC by neutrophils from G-CSF–treated patients.

FcγRIII expression is drastically reduced in G-CSF–treated patients (Table 1). Monomeric polyclonal HuIgG, which does not bind significantly to low-affinity FcγRIII, could totally inhibit the 7A4 MoAb-mediated cytotoxicity.
In contrast, F(ab')2 3G8 MoAb, which interferes with the IgG binding site of FcyRIII, did not inhibit cytotoxicity. In addition, the 7A4 MoAb, a mouse IgG3 that only binds to FcyRI and FcyRII, did not promote any cytotoxicity with freshly isolated FcyRII⁺ neutrophils (data not shown). Thus, our results indicate that FcyRII does not serve as a trigger molecule for ADCC mediated by neutrophils, whether these cells have been activated or not.

The involvement of FcyRI in ADCC parallels its membrane expression on neutrophils isolated from G-CSF-treated cancer patients. It should be noted that neutrophils isolated from the three children with different malignancies and treated with G-CSF for 5 days also exhibited a marked expression of FcyRI and could efficiently lyse neuroblastoma cells. The mechanisms by which in vivo FcyRI expression is strongly increased after G-CSF injections are unknown. G-CSF could directly act on stem cells as in vitro studies have shown that it is able to generate FcyRI⁺ neutrophils from highly purified CD34⁺ bone marrow cells, even in the presence of a neutralizing MoAb directed against IFNγ. In contrast, as shown in our experiments under G-CSF treatment, FcyRII expression is slightly reduced. A marked reduction (10-fold) of FcyRIII expression is also noticed. Between day 1 and day 3 post-G-CSF injection, a decrease of FcyRIII expression on neutrophils derived from healthy individuals who received a single subcutaneous dose of G-CSF has been reported. However, this decrease was transient, as FcyRIII expression reduction was no longer detectable 6 days postinjection. Thus, our data from patients receiving G-CSF for 5 to 12 days suggest that the FcyRIII expression is continually decreased after repeated G-CSF injections.

Our data suggest that the combined use of a BsAb, directed against both FcyRI and GD2, and G-CSF could be a promising approach to target neuroblastoma cells in vivo, as compared with the injection of an anti-GD2 MoAb alone. Two unique advantages in using BsAbs such as the MDX-260 BsAb are showed in the present work. First, its binding to FcyRI is not inhibited by the presence of monomeric or heat-aggregated IgG, in contrast with the whole anti-GD2 murine IgG3 MoAb. Thus, in vivo, its ability to promote GD2⁺ neuroblastoma cell cytotoxicity by neutrophils of G-CSF-treated cancer patients might not be blocked by the presence of circulating monomeric IgG or IgG-containing immune complexes. Second, this BsAb lacks the Fc regions of the parental MoAbs and, thus, does not activate complement cascade. This may prevent the severe side effect observed in all anti-GD2 therapy trials, marked by considerable pain, which can be related to a complement-dependent toxicity of GD2⁺ expressing neurosensitive cells. However, we cannot exclude the possibility that the BsAb-mediated interaction of G-CSF-activated neutrophils with these latter cells could provoke inflammatory reactions and lead, therefore, to the same side effect. Finally, the use of recently developed genetic engineering techniques to produce diabodies directed against human FcyRI and GD2 should permit the development of a second generation of BsAbs, possibly human or humanized, suitable for in vivo therapy in combination with G-CSF.

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