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 cells 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’)_2 22 MoAb abrogated the MDX-260 killing without affecting that of 7A4. The 3G8 MoAb, directed against the FcγRIII 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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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, Neully-sur-Seine, France) for more than 10 days 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. Polymorphonuclear cells (PMNCs) 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. Neutrophils as effector cells were preincubated with 200 IU/mL of G-CSF and morphprep (Nycomed. Oslo, Norway) density gradient was used to enrich neutrophils in vivo stimulated preparations. All the purity of these preparations was over 90% as determined by analytical Superdex 200 chromatography. A major component of this preparation was FcγRIIa, a receptor for IgG Fc. This receptor is expressed on the surface of neutrophils and other leukocytes, and its expression is regulated by the presence of ligands. The purification of neutrophils was monitored by flow cytometry using a specific antibody against FcγRIIa.

The hybridoma B cell lines 7A4 (mouse IgG3, κ, anti-G3) and PS1.1 (IgG3, κ, anti-phosphorylcholine) were provided by Drs D. Beck (CHUV, Lausanne, Switzerland) and M. Herlyn (The Wistar Institute, Philadelphia, PA), respectively. The G3 (IgG2a, κ, anti-G3) and 22 MoAbs 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. The MoAbs were 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 G3 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 10^6/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 (Titerhek 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 FACScan cytometer (Becton-Dickinson, Pont de Claix, France), using the Lysis program.

Complement-dependent cytotoxicity assay. One hundred micro-
liters of LAN 1 cells (2 x 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'Étoile, France) at 37°C for 45 minutes. Cytotoxicity was evaluated using the trypan blue exclusion assay.

**ADCC assay.** Target cells (3 x 10^6 to 10 x 10^6) were incubated for 1 hour at 37°C with 7,400 µBq of ^51^Cr (37 mBq/mL; New England Nuclear DuPont, Bad Homburg, Germany). After two washes in culture medium, 5 x 10^6 [^51^Cr]-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 ^51^Cr minimum release was determined by counting the supernatant of 5 x 10^4 target cells incubated with culture medium only (spontaneous release). The maximum release was estimated by incubating 5 x 10^5 [^51^Cr]-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-FcγR 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 Wallace). The mean value of triplicates was used to calculate the percentage of specific [%Cr] 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 FcγRI⁺ neutrophils or GD2⁺ neuroblastoma cells.** Table 1 shows that neutrophils isolated from G-CSF–treated cancer patients express high levels of FcγRI, as compared with healthy volunteers. In G-CSF–treated patients, expression of FcγRI is significantly increased, whereas FcγRIII is markedly reduced and FcγRII is slightly decreased.

Both the 22-FITC MoAb and the MDX-260 BsAb bind to FcγRI⁺ 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 FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) on Neutrophils Isolated From Cancer Patients Undergoing G-CSF Treatment**

<table>
<thead>
<tr>
<th></th>
<th>G-CSF Patients (n = 8)</th>
<th>Controls (n = 4)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>93 ± 12</td>
<td>11 ± 4</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>FcγRII</td>
<td>120 ± 39</td>
<td>230 ± 39</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>137 ± 89</td>
<td>2,754 ± 39</td>
<td>&lt;.01</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.
TARGETING OF G-CSF–TREATED NEUTROPHILS BY BsAb

Cross-linking of G\textsubscript{D2} to Fc\textgamma{RI} by MDX-260 BsAb. The ability of MDX-260 BsAb to bind to both G\textsubscript{D2} and Fc\textgamma{RI} was investigated by an indirect ELISA using the Fc\textgamma{RI}-\mu fusion protein. Both the MDX-260 BsAb batches were tested. Figure 3 shows that the MDX-260 BsAb simultaneously binds to both G\textsubscript{D2} and Fc\textgamma{RI}. Similarly, the anti-G\textsubscript{D2} parental 7A4 MoAb, a mouse IgG3 that binds human Fc\textgamma{RI}, 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\textsuperscript{'}) × F(ab\textsuperscript{'}) BsAb. This is an expected result, as the 7A4 MoAb is bivalent with respect to G\textsubscript{D2} binding, while 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. [\textsuperscript{51}Cr]-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 (PS1.1) 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 \textmu g/mL. Studies of 10 donors indicated that both antibodies used at this concentration induced about 60% [\textsuperscript{51}Cr]-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 \mu g/mL; data not shown). More than 60% of [\textsuperscript{51}Cr]-specific release was still obtained when using 0.15 \mu g/mL of 7A4 MoAb, whereas the same concentration of MDX-260 BsAb provoked a 35% to 40% toxicity. The other G\textsubscript{D2} cell lines (ACN 75, IMR 32, and HT 144) were also efficiently lysed with the 7A4 MoAb and the MDX-260 BsAb, whereas the G\textsubscript{D2} SW 626 ovarian carcinoma cell line and the G\textsubscript{D2} 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 IFNy (data not shown).

Inhibition of cytotoxicity by HuIgG. High-affinity Fc\textgamma{RI} 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 Fc\textgamma{R}s (Fc\textgamma{RI}, II, and III), whereas monomeric IgG only blocks Fc\textgamma{RI}. 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

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>7A4 MoAb</th>
<th>MDX-260 BsAb</th>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAN 1 (G\textsubscript{D2}\textsuperscript{+})</td>
<td>95 ± 5.4</td>
<td>95 ± 2.9</td>
</tr>
<tr>
<td>ACN 75</td>
<td>89 ± 3.1</td>
<td>62 ± 1.7</td>
</tr>
<tr>
<td>HT 144 (G\textsubscript{D2}\textsuperscript{+})</td>
<td>77 ± 6.6</td>
<td>51 ± 0.2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAN 1 (G\textsubscript{D2}\textsuperscript{+})</td>
<td>80 ± 1.5</td>
<td>77 ± 4.3</td>
</tr>
<tr>
<td>ACN 75</td>
<td>94 ± 8.3</td>
<td>50 ± 2.7</td>
</tr>
<tr>
<td>IMR 32 (G\textsubscript{D2}\textsuperscript{+})</td>
<td>71 ± 3.3</td>
<td>69 ± 6.5</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAN 1 (G\textsubscript{D2}\textsuperscript{+})</td>
<td>81 ± 4.3</td>
<td>54 ± 3.8</td>
</tr>
<tr>
<td>902 B (G\textsubscript{D2}\textsuperscript{+})</td>
<td>9 ± 3.0</td>
<td>10 ± 1.7</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAN 1 (G\textsubscript{D2}\textsuperscript{+})</td>
<td>ND</td>
<td>72 ± 1.8</td>
</tr>
<tr>
<td>SW 626</td>
<td>ND</td>
<td>2 ± 1.3</td>
</tr>
</tbody>
</table>

Data are expressed as percent specific release at an E:T ratio of 100:1 (mean of triplicate ± SD).

Abbreviation: ND, not determined.

Fig 4. Lysis of G\textsubscript{D2}– LAN 1 neuroblastoma cells by neutrophils isolated from a cancer patient receiving G-CSF treatment, at E:T ratios ranging from 100:1 to 125:1 in the presence of 1 \mu g/mL of the 7A4 MoAb, MDX-260 BsAb, PS1.1 MoAb, or medium. Each data point is the mean value of a triplicate ± SD.
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 LAN 1 cells via FcyRI, 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 5). Similarly, the addition of IV.3 or 3G8 F(ab')2 MoAbs, which interfere with FcyRII and FcyRIII IgG binding sites, respectively, did not provoke any inhibition of cell cytotoxicity (Fig 5), thus demonstrating that neither FcyRII nor FcyRIII 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 FcyRI, which is a trigger molecule for ADCC. We show here, using an anti-GD2/anti-FcyRI BsAb (MDX-260), that these neutrophils can efficiently lyse GD2+ cell lines in vitro through the engagement of FcyRI. 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 FcyRI 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 FcyRII and FcyRIII had no inhibitory effect on ADCC. However, the engagement of FcyRII 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 FcyRII (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, FcyRI is probably the only FcyR able to mediate ADCC by activated neutrophils. In addition, the data reported here confirm that FcyRI, and not FcyRIII, is an important trigger molecule for the ADCC exerted by neutrophils derived from G-CSF–treated cancer patients. An anti-FcyRI MoAb, 197, that blocks IgG binding to FcyRI, 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 FcyRI 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.

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

The involvement of FcγRI 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 FcγRI and could efficiently lyse neuroblastoma cells. The mechanisms by which in vivo FcγRI 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 FcγRI* neutrophils from highly purified CD34* bone marrow cells, even in the presence of a neutralizing MoAb directed against IFNγ.18

In contrast, as shown in our experiments under G-CSF treatment, FcγRII expression is slightly reduced. A marked reduction (10-fold) of FcγRIII expression is also noticed. Between day 1 and day 3 post-G-CSF injection, a decrease of FcγRII expression on neutrophils derived from healthy individuals who received a single subcutaneous dose of G-CSF has been reported.11 However, this decrease was transient, as FcγRIII 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 FcγRIII expression is continually decreased after repeated G-CSF injections.

Our data suggest that the combined use of a BsAb, directed against both FcγRI and G02, and G-CSF could be a promising approach to target neuroblastoma cells in vivo, as compared with the injection of an anti-G02 MoAb alone. Two unique advantages in using BsAbs such as the MDX-260 BsAb are showed in the present work. First, its binding to FcγRI is not inhibited by the presence of monomeric or heat-aggregated IgG, in contrast with the whole anti-G02 murine IgG3 MoAb. Thus, in vivo, its ability to promote G02+ 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. This may prevent the severe side effect observed in all anti-G02 therapy trials, marked by considerable pain, which can be related to a complement-dependent toxicity of G02+ 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 diabodies19 directed against human FcγRI and G02 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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