Antidisialoganglioside/granulocyte macrophage–colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcγRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis


Introduction

Antibody-cytokine fusion proteins combine the targeting ability of antibodies with the immune stimulation of cytokines for cancer immunotherapy.1 Studies in mice of antidisialoganglioside (anti-GD2)/interleukin-2 and anti-GD2/lymphotoxin-α fusion proteins (immunocytokines) demonstrated the eradication of hepatic metastases of neuroblastoma2,3 and the induction of protective immunity (immunocytokines) demonstrated the eradication of hepatic metastases of neuroblastoma2,3 and the induction of protective immunity.4 These and other immunocytokine strategies have been based on the stimulation of NK-cell– or T-cell–mediated responses. Polymorphonuclear leukocytes (PMNs) constitute the largest population of white blood cells, and they can mediate ADCC against tumor cells.5-9 Mac-1 (CD11b/CD18), also known as CR3, is a major PMN 2-integrin receptor that, on activation, acquires the ability to bind multiple ligands and to mediate several adhesion-dependent processes, including phagocytosis, superoxide production, and degranulation.10-17

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through distinct signaling pathways. Therefore, we hypothesized that a GM-CSF immunocytokine may mediate greater Mac-1 activation than the antibody component alone and that this would be associated with greater PMN ADCC.

In the present study, we tested the humanized anti-GD2 mAb hu14.18 and fusion protein hu14.18/GM-CSF in PMN ADCC against neuroblastoma cells. Our data demonstrate that hu14.18/GM-CSF significantly increases PMN ADCC compared to hu14.18 alone or mixed with GM-CSF and that ADCC with both hu14.18/GM-CSF and hu14.18 depends on Mac-1 function. Hu14.18/GM-CSF increased ADCC by increasing the expression and activation of Mac-1, adhesion and spreading of neutrophils onto neuroblastoma cells, and azurophil (primary) granule exocytosis.

Materials and methods

Cell culture and reagents

The LA-N-1 neuroblastoma cell line was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gemini-Bio-Products, Calabasas, CA), and 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA) (complete RPMI 1640 medium). Hanks balanced salt solution was from Irvine Scientific. Histopaque-1077 was from Sigma Diagnostics (St Louis, MO). Calcein acetoxymethyl ester (calcein-AM) and propidium iodide, and 1,1′-dioctadecyl-3,3,3,3′-tetramethyl-indodicarbocyanine perchlorate (DiD) were from Molecular Probes (Eugene, OR). Sargramostim leukine (GM-CSF; 250 μg/mL) was reconstituted with sterile water and purchased from Immunex (Seattle, WA).

Humanized anti-GD2 hu14.18 mAb and hu14.18/GM-CSF immunocytokine

Humanized anti-GD2 mAb hu14.18 and hu14.18/GM-CSF fusion proteins (immunocytokine) were prepared at Lexigen Pharmaceuticals (Lexington, MA). Briefly, the original 14.18 V regions were humanized by CDR grafting into human frameworks with extensive homology with the murine sequences. Engineered sequences were introduced as cDNAs into an antibody expression vector already containing the human constant region genes for the light and the heavy chains or for heavy chain fused in-frame with the cDNA encoding human GM-CSF. The vector was introduced into the supernatant were subjected to density separation using Histopaque-1077 (Sigma Diagnostics) and centrifugation. PMNs were collected from the supernatant. PMNs were 95% to 99% pure and 97% to 99% viable, as determined by flow cytometry forward and side scatter and propidium iodide exclusion.

ADCC assay

ADCC was quantified by measuring retained calcine fluorescence in target cells with Digital Image Microscopy Scanning (DIMSCAN) as previously described. Neuroblastoma cells were detached with Puck saline A and 1 mM EDTA (Puck EDTA), washed, and resuspended in complete RPMI 1640 medium. Calcine-AM was added to achieve a final concentration of 5 μg/mL, and cells were incubated at 37°C for 30 minutes in the dark. After incubation, cells were washed and resuspended in complete RPMI 1640 medium, counted using trypan blue to identify viable cells, and added to microwells (4000 cells in 100 μL per well; 96-well Falcon 3072 plates, Becton Dickinson, Franklin Lakes, NJ). Appropriate concentrations of effector cells, hu14.18 antibody, GM-CSF, and hu14.18/GMCSF were prepared in complete RPMI 1640 medium just before plating, and 6 replicate wells for each condition were set up with a final volume of 200 μL. Each plate included 6 wells of neuroblastoma cells alone as a control. Plates were incubated at 37°C for 4 hours, and retained fluorescence was quantified by DIMSCAN. Cytotoxicity was expressed as a tumor cell viability index. Mean tumor cell viability index ± SD was calculated for each condition from 6 replicate wells.

Flow cytometry

Did (5 μM) was added to cultured LA-N-1 neuroblastoma cells for 12 hours and was followed by the detachment of cells with Puck EDTA. PMNs and neuroblastoma cells were placed in 96-well plates with an effector-target ratio of 20:1 (200000 PMNs and 10000 neuroblastoma cells in 100 μL complete RPMI 1640 medium per well). ADCC was initiated by adding hu14.18 or hu14.18/GM-CSF to a final concentration of 5 μg/mL. After desired periods of incubation at 37°C, anti-CD11b-FITC, anti-CD63-FITC (or -PE) CLBGran/12 (Immunotech, Miami, FL); anti-CD11b 2LMP19c (Dr K. Pulford, Oxford, United Kingdom); and anti-β2-activation reporter epitope mAb24 (Dr N. Hogg, London, United Kingdom).

Monoclonal antibodies

The following mAbs were used: anti-CD32 IV.3 Fab, anti-CD16 3G8 (Fab) (Medarex, West Lebanon, NH); anti-CD18 R3.3, anti-CD11a R7.1, anti-CD11b LM2/L, anti-CD11c CBR-p50/4G1, anti-CD11c 3.9 ( Biosource International, Camarillo, CA); anti-CD18 7E4, anti-CD11a 25.3, anti-CD11c BU15, anti–CD63-FITC (or -PE) CLBGran/12 (Immunotech, Miami, FL); and anti-CD11b 2LMP19c (Dr K. Pulford, Oxford, United Kingdom; and anti-β2-activation reporter epitope mAb24 (Dr N. Hogg, London, United Kingdom).
Eppendorf 5415 C microcentrifuge, Brinkmann Instruments, Westbury, NY), and resuspended in PBS solution (100 µL) with secondary goat antimouse PE-conjugated F(ab')2 (10 µg/mL final concentration; Immunotech). After 20-minute incubation at room temperature, cells were washed and resuspended in 100 µL PBS solution with FITC-conjugated anti-CD11b or anti-CD63 mAb (10 µg/mL final concentration) for 20 minutes at room temperature. Finally, cells were diluted to 600 µL with PBS solution, gently transferred to flow cytometry tubes, and analyzed within 15 minutes.

Argon 488-nm and HeNe 633-nm lasers were colinearly aligned and used to excite FITC, PE, and DiD, respectively. Samples stained with each dye separately were run to determine the level of electronic compensation required with a Beckman-Coulter (Hialeah, FL) band-pass filter set (525 ± 20 nm; 575 ± 20 nm, and 675 ± 20 nm) to achieve separation of emitted signals from the 3 dyes. Ten thousand events were analyzed for each specimen, which was derived from 4 replicate microwells. Each set of experiments was performed 3 times.

**Electron microscopy**

Neuroblastoma, antibody, and PMN mixtures were set up in 96-well plates as described above. Cell pellets were prepared after indicated time intervals by combining cells from 4 replicate wells per condition and centrifuging them for 10 minutes (3000 rpm; Beckman Microfuge E). Cells then were fixed with 2% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide in PBS, dehydrated by a graded series of ethanol, and embedded in Epon-812. Polymerization was carried out in a 60°C oven for 48 hours. After examining semithin sections by light microscopy, ultrathin sections were cut, mounted on collodion one-hole grids, and stained with uranyl acetate and lead citrate. Ultrastructural images were examined and photographed using a Philips CM12 electron microscope. Semiquantitative analysis of PMN spreading was performed by counting electron microscopy images of 10 tumor cells and identifying those with a single PMN covering at least 30% of target cell surface.

**Data analyses**

Flow cytometry data were analyzed with EXPO Analysis software (Beckman-Coulter). We used Excel (Microsoft, Redmond, WA) to analyze DIMSCAN ADCC data to calculate the mean ± SD for 6 replicate wells. Data are from 1 of 4 experiments that gave similar results.

**Results**

**PMN ADCC is greater with hu14.18/GM-CSF than with hu14.18 alone or mixed with GM-CSF**

PMN ADCC activity of hu14.18, hu14.18 mixed with GM-CSF, and hu14.18/GM-CSF was determined using LA-N-1 neuroblastoma cell targets at levels achieved in serum in phase 1 studies of the closely related human–mouse chimeric 14.18 antibody (Figure 1).27-30 Pilot experiments showed that GM-CSF alone (1000 ng/mL) did not induce PMN cytotoxicity but did increase PMN ADCC when added to hu14.18 (5 µg/mL), with the increase reaching a plateau at 10 ng/mL GM-CSF (data not shown). Hu14.18 alone induced maximal cytotoxicity at 1 µg/mL, with 75.3% ± 4.9% tumor cells remaining viable at 4 hours. Addition of soluble GM-CSF (100 ng/mL) significantly increased ADCC by hu14.18, with 58.3% ± 2.2% tumor cells viable at 2.5 µg/mL (P < .001, t test). Hu14.18/GM-CSF mediated greater ADCC than hu14.18 alone (P < .001, t test) or mixed with GM-CSF (P < .001, t test), with only 22.6% ± 2.3% of cells viable at 2.5 µg/mL. Thus, though hu14.18 and hu14.18/GM-CSF bind equally well to neuroblastoma cells (see “Materials and methods”), the latter is more effective in mediating PMN ADCC.

**FcyRII and Mac-1 are required for PMN ADCC with hu14.18/GM-CSF**

To identify Fcy receptors required for PMN ADCC mediated by hu14.18 and hu14.18/GM-CSF, we used IV.3 anti-FcγRII F(ab')2 blocking mAbs (Figure 2A). ADCC mediated by hu14.18 alone or with added GM-CSF (100 ng/mL) was dependent on FcγRI (CD32) and FcγRII (CD16). In contrast, hu14.18/GM-CSF, which mediated the strongest ADCC, required only CD32. FcγRI (CD64) was not detectable on freshly isolated PMNs or after 4-hour incubation with GM-CSF or hu14.18/GM-CSF (data not shown).

Function-blocking mAbs against the common β-subunit (CD18) and against each α-subunit (CD11a, CD11b, and CD11c) were used to examine the requirement for β2-integrin receptors in PMN ADCC. The blocking mAbs 7E4 against CD18 and 2LPM19C against CD11b completely abrogated ADCC mediated by hu14.18/GM-CSF (Figure 2B) and hu14.18, with or without GM-CSF (data not shown). Blocking antibodies against other α-subunits (CD11a and CD11c) did not inhibit cytotoxicity. Thus, the increased ADCC with hu14.18/GM-CSF compared with hu14.18 does not require CD16 but is absolutely dependent on CD32 and Mac-1 (CD11b/CD18).

**Spreading and adhesion by PMN on target cells and cytolyis is greatest with hu14.18/GM-CSF and requires Mac-1**

Cell-cell interactions between PMNs and tumor cells in ADCC were visualized with electron microscopy at 30 minutes and 2 hours after the start of ADCC. There was no or minimal spontaneous interaction between PMN and neuroblastoma cells in the absence of hu14.18 and hu14.18/GM-CSF (Figure 3A-B). In the presence of hu14.18 (Figure 3C-D) and hu14.18/GM-CSF (Figure 3E-F), PMN pseudopodia adhered to the surfaces of neuroblastoma cells. However, spreading and tight adhesion was evident with hu14.18/GM-CSF but not with hu14.18. In nearly all conjugates formed at 30 and 120 minutes with hu14.18/GM-CSF, PMNs
ADCC were Mac-1 dependent, we examined cell surface expression of Mac-1 during PMN ADCC with 2-color flow cytometry analysis (Figure 7). Degranulation stimulated by hu14.18 with or without GM-CSF and by hu14.18/GM-CSF was associated with mAb24 + PMNs and was almost absent in the mAb24 - population (Figure 7A). Hu14.18/GM-CSF induced a time-dependent increase in CD63 expression by mAb24 + PMNs that was significantly higher than that induced by hu14.18 with or without GM-CSF from 30 to 120 minutes (P < .001, ANOVA). Anti-FcγRII (CD16) and anti-CD11b, but not anti-FcγRIII (CD32) function-blocking mAbs, abrogated CD63 expression mediated by hu14.18/GM-CSF (Figure 7B). GM-CSF alone did not increase conjugate formation at any time in comparison with control PMNs alone (P > .05, ANOVA). Similar results were obtained with 4 different effector-target ratios ranging from 1:1 to 25:1 (data not shown).

Degranulation is greatest with hu14.18/GM-CSF and requires FcγRII and activated Mac-1

CD63 is a marker of azurophil (primary) granules, and exocytosis results in the expression of CD63 on the cell surface because of granule fusion with the cell membrane. The relationship between Mac-1 activation (mAb24 + PMN) and CD63 expression during PMN ADCC was examined using 2-color flow cytometry analysis (Figure 7). Degranulation stimulated by hu14.18 with or without GM-CSF and by hu14.18/GM-CSF was associated with mAb24 + PMN and was almost absent in the mAb24 - population (Figure 7A). Hu14.18/GM-CSF induced a time-dependent increase in CD63 expression by mAb24 + PMNs that was significantly higher than that induced by hu14.18 with or without GM-CSF from 30 to 120 minutes (P < .001, ANOVA). Anti-FcγRII (CD16) and anti-CD11b, but not anti-FcγRIII (CD32) function-blocking mAbs, abrogated CD63 expression mediated by hu14.18/GM-CSF (Figure 7B). GM-CSF alone did not increase conjugate formation at any time in comparison with control PMNs alone (P > .05, ANOVA). Similar results were obtained with 4 different effector-target ratios ranging from 1:1 to 25:1 (data not shown).
Discussion

This study demonstrated that a humanized anti-GD2/GM-CSF immunocytokine, hu14.18/GM-CSF, is more effective in mediating PMN ADCC than the parent anti-GD2 mAb hu14.18 alone or mixed with GM-CSF and that ADCC under all conditions required functional Mac-1 (CD11b/CD18). Electron microscopy revealed greater Mac-1–dependent PMN adhesion, spreading, and cytolysis with hu14.18/GM-CSF than with hu14.18. Flow cytometry showed that increased expression and activation of Mac-1 occurred during PMN ADCC with mAb hu14.18 alone but that this was significantly less than with GM-CSF, GM-CSF mixed with hu14.18, and hu14.18/GM-CSF, indicating the importance of GM-CSF in regulating Mac-1. Even though the latter conditions equally enhanced the expression and activation of Mac-1, hu14.18/GM-CSF caused the highest and most sustained azurophil granule exocytosis, which was Mac-1 and FcγRII dependent. These experiments demonstrate for the first time the importance of enhanced expression and activation of Mac-1 for PMN ADCC against tumor cells. Additionally, they suggest that enhancing azurophil granule exocytosis is critical for maximizing PMN ADCC.

The first step in PMN ADCC is the interaction of target-bound antibody with PMN FcR. FcγRII and FcγRIII were previously reported to be necessary for PMN ADCC against neuroblastoma cells with the 3F8 anti-GD2 murine mAb. However, PMN ADCC against Burkitt lymphoma cells (Raji cell line) was shown to require only FcγRII using F(ab′)2 reagents to block FcR. Our experiments demonstrated that both receptors are required for PMN ADCC with hu14.18, whereas only FcγRII is required with hu14.18/GM-CSF. We used IV.3 F(ab′)2 to test FcγRII and 3G8 F(ab′) to test FcγRIII requirements, thus avoiding potential interactions of the Fc portion of the blocking mAbs with FcR. We found that the whole 3G8 anti-FcγRIII mAb used in the earlier study of FcRs in PMN ADCC nearly completely abrogated ADCC mediated by hu14.18/GM-CSF (data not shown), indicating that the whole 3G8 mAb has a nonspecific effect. FcγRII use by hu14.18 with or without GM-CSF, but not by hu14.18/GM-CSF, may be the result of one or more of the following: (1) the GM-CSF component of hu14.18/GM-CSF may alter the tertiary structure of the Fc portion, decreasing its affinity for FcγRIII; (2) simultaneous cross-linking of the FcγRII and GM-CSF receptors by hu14.18/GM-CSF may overcome a requirement for FcγRIII signaling; (3) shedding of FcγRIII from the PMN

Table 1. Semiquantitative analysis of PMN spreading over neuroblastoma cells during ADCC

<table>
<thead>
<tr>
<th>Condition</th>
<th>Tumor cells with attached and spread PMNs (%)</th>
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<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>mAb hu14.18</td>
<td>0</td>
</tr>
<tr>
<td>IC hu14.18/GM-CSF</td>
<td>80</td>
</tr>
<tr>
<td>IC + anti-CD18</td>
<td>0</td>
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<tr>
<td>IC + anti-CD11b</td>
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Conditions are the same as indicated in Figures 3 and 4. Percentages are of tumor cells per 10 counted for which at least 30% of the cell surface was covered by a single PMN. Analysis was performed using electron microscopy as described in Figures 3 and 4.
The surface may be increased by GM-CSF in the hu14.18/GM-CSF-CSF immunocytokine. With function-blocking mAbs, we showed that Mac-1 is required for PMN ADCC mediated by both hu14.18 and hu14.18/GM-CSF. This is in agreement with other reports that Mac-1 is required for PMN ADCC against tumor cells. This is the first demonstration that PMN ADCC with an antibody/GM-CSF immunocytokine requires Mac-1 function. Electron microscopy of PMN ADCC with hu14.18/GM-CSF or with hu14.18 revealed the greatest adhesion, spreading, and tumor cell lysis with the immunocytokine. When anti-CD18 or anti-CD11b-blocking mAbs were included with hu14.18/GM-CSF, PMNs were tethered to target cells but did not adhere, spread, or cause cytolysis. Mac-1-mediated

**Figure 4.** Effect of function-blocking mAbs against Mac-1 (CD11b/CD18) on spreading, adhesion, and cytolysis by PMN with hu14.18/GM-CSF. ADCC with hu14.18/GM-CSF was as described in Figure 3 except that function-blocking mAbs were added just before the addition of hu14.18/GM-CSF. ADCC was performed in the presence of anti-CD18 7E4mAb (10 μg/mL) for (A) 30 minutes and (B) 120 minutes or anti-CD11b 2LPM19C mAb (10 μg/mL) for (C) 30 minutes and (D) 120 minutes. Electron micrographs were taken with an original magnification of ×5400.

**Figure 5.** Expression and activation of Mac-1 during PMN ADCC. (A) PMNs and DiD-labeled neuroblastoma cells were incubated alone (control), with hu14.18, or with hu14.18/GM-CSF, and flow cytometry analyses were performed at 30, 60, and 120 minutes. In the example shown, expression of CD11b and the β2-integrin activation epitope were analyzed 60 minutes after the initiation of ADCC with FITC–anti-CD11b (the nonblocking Bear-1 mAb) and mAb24 (PE–anti-mouse IgG), respectively. (B) The percentage of mAb24-positive cells in PMN subpopulations expressing high or low levels of CD11b after 60 minutes of ADCC was determined by gating on these distinct subsets. Binding index (% positive cells × mean fluorescence channel/100) for (C) CD11b and (D) mAb24 was calculated at indicated times. Significant differences compared to controls are indicated by "**" for P < .01 and "***" for P < .001 (ANOVA). Data are from 1 of 3 experiments that provided similar results.

**Figure 6.** PMN–tumor cell conjugate formation. PMNs were incubated with DiD-labeled neuroblastoma cells alone (control), GM-CSF alone, hu14.18 with or without GM-CSF, or hu14.18/GM-CSF immunocytokine for 30, 60, 90, and 120 minutes. (A) In this example, the expression of CD11b by PMNs is shown after 60 minutes with an FITC–anti-CD11b mAb, and data are presented as dual-color diagrams. (B) PMN–tumor cell conjugates were identified as CD11b+DiD+ events and are represented as the number per 10,000 events at the indicated times. Data are from 1 of 5 experiments that gave similar results.
focal adhesion contacts also have been described recently for PMN ADCC with an anti-HER-2/neu murine mAb and with an anti-FcεRI/anti-HER-2/neu bispecific mAb.3 These experiments indicate that an essential and important function of Mac-1 is to mediate strong adhesion and spreading of PMN onto tumor cells.

Flow cytometry showed an increase in expression and activation of Mac-1 during PMN ADCC with a hu14.18 alone and an even greater increase with GM-CSF; GM-CSF mixed with hu14.18, and hu14.18/GM-CSF (Figure 5). This indicates the importance of antibody and GM-CSF in regulating Mac-1. Functionally active Mac-1 was identified with mAb24, which recognizes an epitope within the I domain of the β2-integrin α-subunit when it binds Mg2+.35,36 Two-color staining of PMN with mAb24 and Bear-1 anti-CD11b mAbs showed Mac-1 activation predominantly in CD11bhigh cells, though the up-regulation of CD63, the latter induced greater CD63 expression than the mixture, GM-CSF alone did not cause azurophil granule exocytosis compared to control, untreated PMNs. The hu14.18/GM-CSF immunocytokine induced PMN degranulation only in the presence of the target cells, and this was FcγRII and Mac-1 dependent, indicating that azurophil granule release in ADCC requires PMN attachment, adhesion, and spreading on target cells. Sustained Mac-1–mediated PMN adhesion and spreading may also be important for ADCC by providing maximal concentrations of neutrophil granule contents at the tumor cell surface and an optimal pH for enzymatic activity of primary granule proteases.37 Thus, our data suggest that mAb24–CD63+ PMNs are the cytolytic effectors in PMN ADCC. Supporting this model is a previous report that PMN azurophil granule exocytosis is required for PMN ADCC against neuroblastoma cells with the anti-GD2 chimeric mAb ch14.18 and that this is enhanced by the addition of GM-CSF.38

Although our data demonstrate that Mac-1 mediates adhesion, spreading, and azurophil granule exocytosis once PMN–tumor cell conjugates have been established by antibodies, it is not clear how Mac-1 contributes to these important events. Ligands for Mac-1 on neuroblastoma cells are unknown. We found that 10 neuroblastoma cell lines expressed little or no ICAM-1, a known ligand for Mac-1, by flow cytometry (data not shown), and this is in accord with other reports.40 Therefore, Mac-1 may bind other unknown molecule(s) or may transmit signals from GPI-linked receptors given that cooperation with Mac-1 through the lectinlike site on CD11b has been reported for CD66b.41 Indeed, Mac-1 activation through specific binding to the lectinlike domain of the COOH-terminus of CD11b is involved in the antitumor activity of the soluble β-glucans lentatin and schizophyllan.41

We conclude that Fc and GM-CSF receptor triggering increase the expression and activation of Mac-1, which is necessary for strong adhesion and spreading of PMN on target cells and for azurophil granule exocytosis. We propose that the hu14.18/GM-CSF immunocytokine increases PMN ADCC by enhancing Mac-1–dependent azurophil exocytosis. Further studies exploring additional means of increasing Mac-1 function and identifying its ligand(s) on tumor cells will be important for the development of antibody and immunocytokine-based therapies for neuroblastoma and other tumors.

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