Differences in CD33 Intensity Between Various Myeloid Neoplasms

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Key Words: CD33 intensity; Quantitative flow cytometry; Gemtuzumab ozogamicin; Mylotarg; Acute myeloid leukemia; Myelodysplastic syndrome; Myeloproliferative disorder; Chronic myelogenous leukemia; Monoclonal antibody therapy

Abstract

We measured the concentration of CD33 antigen on the surface of cells in 315 bone marrow (BM) samples and 114 corresponding peripheral blood (PB) samples from patients with various leukemias (acute myeloid leukemia [AML], chronic myelogenous leukemia [CML], myelodysplastic syndrome [MDS]) and from control subjects. Overall CD33 intensity in total CD33+ cells was significantly higher in BM than in PB. CD33 intensity in total BM CD33+ cells differed significantly with the type of disease. The median number of CD33 molecules per cell was highest in AML, followed by MDS, CML, and control subjects and lowest in MPD. When only CD34+/CD33+ cells were examined, CD33 molecules per cell were highest in CD34+ cells in AML and lowest in MPD (P = .027). Patients with AML or MDS younger than 60 years had significantly higher intensity of CD33 expression on CD34+ cells than patients 60 years or older. Levels of CD33 intensity did not correlate with cytogenetics in patients with AML or MDS. There was no correlation between CD33 intensity and response to therapy or overall survival in 35 patients treated with protocols including Mylotarg. These data demonstrate variation in CD33 intensity between various leukemias.

With the advent of monoclonal antibody (MoAb) therapy for leukemia, knowledge about the distribution and concentration of the antigens targeted by such antibodies in different cell populations is becoming crucial. One of the antigens targeted by MoAb therapy is CD33, a 67-kd transmembrane cell-surface glycoprotein1 that has 2 immunoglobulin-like domains and 2 cytoplasmic tyrosine residues. Gemtuzumab ozogamicin (Mylotarg; CMA-676), an anti-CD33 calicheamicin conjugate, recently was approved for treatment of patients with relapsed acute myeloid leukemia (AML) older than 60 years of age.2-5 Upon binding with the antibody, CD33 undergoes modulation (internalization), permitting the delivery of a cytotoxic agent bound to the antibody to the interior of the target cells.6-9 CD33 is found on cells committed to the myeloid lineage, myeloid leukemic blasts, and mature monocytes but not normal pluripotent hematopoietic stem cells.10-13 Its concentration on these cells, however, is unknown. Determining the level of antigen expression may provide some insight into the possibility that a higher intensity of antigen on the surface of cells may imply higher binding of therapeutic antibody and better delivery of conjugated chemotherapy.

Quantitative flow cytometry (QFC), using bead standards, coupled with the QuantiCalC software (Verity Software House, Topsham, ME), permits measurement of the concentration of antigens on the surface of different blood cell populations.14-18 Therefore, the purpose of this study was to compare the level of CD33 expression measured using QFC on WBCs in bone marrow (BM) and peripheral blood (PB) samples obtained from patients with AML, chronic myelogenous leukemia (CML), other myeloproliferative disorders (MPDs), or myelodysplastic syndrome (MDS) and from control subjects. In particular, we studied...
whether CD33 antigen density is heterogeneous by site of specimen and disease type.

**Materials and Methods**

**Patients and Samples**

From August 2000 to May 2001, we prospectively examined CD33 intensity on the surface of WBC populations in 315 consecutive BM samples and 122 corresponding PB samples obtained from patients with various leukemias, including AML (BM, 100; PB, 24), CML (BM, 59; PB, 24), MPD other than CML (BM, 5; PB, 5), MDS (BM, 135; PB, 53), and control subjects (BM, 16; PB, 16). None of the patients studied were receiving any therapy that included anti-CD33 (Mylotarg). Approximately 50% of patients with AML were treated previously, but none were receiving any kind of therapy at the time of analysis. None of the AML cases were a transformation of an abnormal blood count (hemoglobin value, <12 g/dL; or neutrophil count, <1,500/µL [<1.5 × 109 /L]; or WBC count, >10,000/µL [>10.0 × 109 /L] or <4,000/µL [<4.0 × 109 /L]; or platelet count, <150 × 109 /µL [<150 × 109 /L]) documented to be present for at least 1 month before initial examination at the M.D. Anderson Cancer Center, Houston, TX.

Patients with MDS were classified based on the French-American-British classification. The classification was as follows: refractory anemia or refractory anemia with ringed sideroblasts, 25.2%; refractory anemia with excess blasts, 31.9%; refractory anemia with excess blasts in transformation, 23.7%; and chronic myelomonocytic leukemia, 20.0%. Four of 5 patients with MPD had myelofibrosis, and 1 had polycythemia vera. We obtained normal control cells from the BM and PB from patients with normal morphologic features who underwent an evaluation for lymphoma or solid tumor staging. All normal samples were obtained before the patients received any therapy. All samples were obtained under institutional review board–approved protocols, and all patients gave written informed consent. The diagnosis in each case was established using standard morphologic, immunologic, and molecular evaluation. Cytochemical staining for myeloperoxidase, nonspecific esterase, and periodic acid–Schiff also was performed when appropriate. Complete immunophenotyping and cytogenetic studies were performed on all cases of acute leukemia and MDS.

**Quantification of CD33 Intensity**

Approximately 2 × 10⁶ cells from each sample were divided into 2 equal aliquots containing 1 × 10⁶ cells each. One aliquot was stained with a phycoerythrin (PE)-labeled IgG1 MoAb in combination with an allophycocyanin (APC)-labeled IgG1 MoAb to serve as an isotypic control. The remaining 1 × 10⁶ cells were stained with a PE-labeled CD33 MoAb (fluorescence/protein ratio, 1:1; Becton Dickinson, Franklin Lakes, NJ) in combination with an APC-labeled CD34 MoAb (Becton Dickinson). After the cells were incubated with the antibodies at 4°C for 20 minutes, RBCs were lysed with ammonium chloride for 10 minutes and then washed with 1× phosphate buffered saline plus 0.1% sodium azide; the washed cells were then fixed with 1% paraformaldehyde.

A set of 4 microbeads (Quantum Simply Cellular, FCSC, San Juan, Puerto Rico) having different calibrated binding capacities of goat antimouse IgG on their surface was used for standardization before acquisition of samples. The beads were incubated with the same anti-CD33 MoAb (BD Biosciences, San Jose, CA) used on patient samples. Ten thousand events were acquired from each tube using a flow cytometer (FACSCalibur, BD Biosciences), and data were analyzed using a software program (CellQuest, Becton Dickinson). The QuantiCALC software program was used to convert flow cytometry data on CD33 in terms of antibodies molecules per cell. Cells were considered positive if they showed 1 log or higher staining above the isotype control.

To test reproducibility of the quantification, 39 samples were tested for CD33 intensity in duplicate. Each sample was stained and analyzed at a different time in a blinded manner by the same person. When we compared the results of CD33 intensity from these duplicated samples, we found no significant difference between the 2 measurements (P = 1.0; sign test), confirming the high reproducibility of the assay.

**Statistical Analysis**

Comparison of CD33 intensity in BM and PB samples was performed using the Wilcoxon matched-pair test. The Spearman rank correlation test was used to correlate with WBC count, platelet count, and hemoglobin value. Group comparison was performed using the Kruskal-Wallis test, which becomes the t test when only 2 groups are compared. The Kaplan-Meier method was used for the survival curve to determine the significance of CD33 intensity in Mylotarg-treated patients.

**Results**

**Differences in CD33 Intensity Between PB and BM**

CD33 intensity was measured in 315 BM samples and 122 corresponding PB samples obtained from patients having various hematologic disorders. The number of...
samples for each disorder is listed in Table 1. We found that the overall CD33 intensity in all CD33+ cells was higher in BM samples than in PB samples when all of the samples were considered. The mean CD33 intensity in BM samples measured in terms of molecules per cell was 7,200 (range, 6,200-8,200), while that in PB samples was 6,100 (range, 4,600-7,600), with a P value of .001 \(P<.001\) (Figure 1A). This was true even when we gated polymorphonuclear cells only \((P<.001)\) (Figure 1B). Gating on CD34+ cells also demonstrated lower intensity in PB samples \((P=.004)\) (Figure 1C).

### Differences in CD33 Intensity Between Myeloid Diseases

The number of CD33 molecules per cell of CD33+ cells in BM samples differed significantly with the type of disease \((P<.001)\) (Figure 2A). Specifically, the number of CD33 molecules per cell was highest in AML cases, followed by MDS cases, CML cases, control subjects, and MPD cases (Table 1, Figure 2A). There was no significant difference in CD33 intensity between previously treated and previously untreated AML cases \((P=.45)\). When only CD34+ cells were examined, the CD33 level was highest in AML cases, followed by control subjects, CML cases, MDS cases, and MPD cases \((P=.027)\) (Table 1, Figure 2B). In addition, when we compared CD33 intensity in polymorphonuclear cells in bone marrow, it was high in AML, MDS, and CML cases and lower in MPD and control subjects, although the difference was not statistically significant \((P=.20)\) (Figure 2C). Similar results were obtained when we studied CD33 intensity in PB samples. Total CD33+ cells (Figure 3A), CD34+ cells (Figure 3B), and polymorphonuclear cells (Figure 3C) showed a similar pattern. However, unlike bone marrow, CML cases differed in the polymorphonuclear cell region in peripheral blood (Figure 3C) and showed the highest level of CD33 expression.

### Clinical Correlation

Patients with AML or MDS who were younger than 60 years of age had significantly higher CD33 intensity on CD34+ cells than did those who were 60 years of age or older \((P=.01)\) (Figure 4A). However, the level of CD33 intensity did not correlate with cytogenetics or WBC count in these patients. Thirty-five previously untreated patients with AML and MDS in whom CD33 expression was quantitated received treatment on protocols that included Mylotarg. Although the follow-up duration in these patients was short, when the manuscript was prepared, there was no obvious correlation between CD33 intensity and achievement of complete remission or overall survival (Figure 5).

### Discussion

CD33 is an important cell-surface marker that has been used for diagnosing AML.\(^6\)\(^,\)\(^19\)-\(^21\) However, its expression on AML cells and those of other MDSs and MPDs has not been characterized in a quantitative manner. Determining the level of CD33 expression on the surface of cells may have clinical implications when patients receive treatment on a regimen that includes an anti-CD33 agent. Conceivably, cells having a higher CD33 intensity have a greater chance of capturing anti-CD33 agents, internalizing the antibody, and conjugating therapy. Quantification of surface antigens has become easier using the recently introduced QFC method with bead standards used for calibration.\(^14\)-\(^18\),\(^22\) For example, Quantum Simply Cellular is a mixture of 4 highly uniform microbead populations of the same size that have varying capacities to bind to mouse monoclonal IgG antibodies depending on their size. These beads, when labeled with anti-CD33 PE, have the same spectral properties of cells labeled with the same antibody and, therefore, provide an accurate compensation standard that will cover the intensity range of different cells.\(^14\)-\(^18\) A calibration curve then is made using the QuantiCALC software program to illustrate the relationship between the labeled microbeads and the minimum detectable antibody.

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**Table 1**

<table>
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<tr>
<th>Diagnosis</th>
<th>No. of Samples</th>
<th>Total CD33+</th>
<th>CD33+/CD34+</th>
<th>No. of Samples</th>
<th>Total CD33+</th>
<th>CD33+/CD34+</th>
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<td>Peripheral Blood</td>
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<td>AML</td>
<td>100</td>
<td>10,380 (709-54,894)</td>
<td>9,482 (406-63,875)</td>
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<td>9,175 (421-85,452)</td>
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<td>MDS</td>
<td>135</td>
<td>6,671 (493-53,791)</td>
<td>4,643 (295-51,176)</td>
<td>53</td>
<td>5,339 (440-44,810)</td>
<td>5,183 (100-62,750)</td>
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<td>CML</td>
<td>59</td>
<td>4,410 (801-16,108)</td>
<td>8,154 (1,414-19,750)</td>
<td>24</td>
<td>4,478 (866-12,030)</td>
<td>8,386 (1,075-31,995)</td>
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<td>MPD</td>
<td>5</td>
<td>2,295 (666-4,279)</td>
<td>3,138 (787-6,026)</td>
<td>5</td>
<td>1,903 (632-3,975)</td>
<td>2,667 (911-4,756)</td>
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<tr>
<td>Control subjects</td>
<td>16</td>
<td>2,997 (859-5,137)</td>
<td>8,154 (1,414-19,750)</td>
<td>16</td>
<td>2,336 (897-3,981)</td>
<td>5,686 (1,342-21,500)</td>
</tr>
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AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; MPD, myeloproliferative disorder.

\(\text{Data are given as mean molecules per cell (range).}\)
Figure 1. Increased CD33 intensity in bone marrow cells compared with peripheral blood for all CD33+ cells (A; P = .001), polymorphonuclear cells (PMN) alone (B; P = .0003), and CD34+ cells alone (C; P = .004). Square, mean; rectangle, ± 1.00 SE; error bars, ± 1.96 SE.

Figure 2. Comparison between various acute and chronic myeloid leukemias and myelodysplastic syndrome (MDS) in CD33 expression in all CD33+ bone marrow (BM) cells (A; P = .0001), CD34+ cells (B; P = .027), and polymorphonuclear cells (PMN) (C; P = .20). Patients with acute myeloid leukemia (AML) had the highest intensity of CD33 in BM cells. Square, mean; rectangle, ± 1.00 SE; error bars, ± 1.96 SE; CML, chronic myelogenous leukemia; MPD, myeloproliferative disorder.
number (antibody-binding capacity), which subsequently makes it possible to quantitate the number of antigen sites.

We demonstrated that overall CD33 intensity varied significantly \((P = .001)\) between BM and PB samples (Figure 1A); specifically, CD33 intensity in BM samples was higher than that in PB samples. This was not due to a higher percentage of immature cells in the BM samples, however, because on gating CD34+ cells alone, similar results were obtained. Furthermore, a difference in antigen intensity between BM and PB samples has been observed using other antigens such as CD20.\(^{23}\) The reason for this difference is not known. In addition, the CD33 intensity in CD33+ cells in BM samples in our study differed significantly with the type of disease \((P < .001)\). As shown in Figure 2, the number of CD33 molecules per cell was highest in AML cases, followed by MDS cases, CML, control subjects, and MPD cases.
When only CD34+ cells were examined, the CD33 intensity was highest in AML cases, followed by control subjects, CML cases, MDS cases, and MPD cases ($P = .027$) (Figure 2B). Also, when we gated on polymorphonuclear cells only, there was no statistical difference ($P = .20$) between the various diseases. Finally, the CD33 intensity in PB samples was similar in CD33+ and CD34+ cells but differed in the polymorphonuclear cell region in CML cases.

When CD33 intensity was compared in patients with AML or MDS who were younger than 60 years of age, it was significantly higher on CD34+ cells than in patients who were 60 years of age or older ($P = .01$). No correlation was found with cytogenetics or WBC count in these patients. There also was no correlation between CD33 intensity and response to therapy or overall survival in the 35 patients treated with protocols that included Mylotarg. However, the follow-up duration in these patients was short.

These data demonstrate the variation in CD33 intensity among various leukemias. Further study is needed to determine whether this variation has any effect on Mylotarg-based therapy. Furthermore, therapy for various diseases using Mylotarg may result in variations in response to it depending on the CD33 intensity in each case.

References


