Masking of Neutrophil Surface Lectin-Binding Sites in Chronic Myelogenous Leukemia (CML)

By Robert N. Taub, Michael A. Baker, and K. R. Madyastha

We have conducted experiments to determine whether alterations in cell surface oligosaccharide residues might be related to altered granulocyte function in chronic myeloid leukemia (CML). Peripheral blood neutrophils from patients with Philadelphia chromosome-positive CML, patients with benign leukocytosis, and 18 normals were tested for leukoagglutination against concanavalin A (Con-A), peanut lectin (PNA), and limulin (LIM). Cells were also assayed for their ability to adhere to column-packed nylon fibers. Compared to normal, neutrophils from patients with CML (WBC 25-600,000/cu mm) showed reduced or absent agglutination with PNA, and increased reactivity to LIM. CML cells also showed distinctly decreased adhesiveness, with up to 50% passing through the nylon-fiber column; normals showed less than 5% nonadherent cells. The effluent subpopulation of less adhesive CML cells differed from those of the unfraccionated suspension in showing a greater reduction of reactivity with PNA and Con-A and increased agglutination by limulin. In vitro treatment of granulocytes of CML patients with vibrio cholera neuraminidase (VCN) partially reversed the defect in adherence toward normal. VCN treatment of normal cells had little effect. These data suggest the possibility that in CML, sialic acid or hypersialylated glycoproteins may mask cell surface receptors that may mediate granulocyte adhesion. Similar inhibition of receptors involved in neutrophil margination and emigration from capillaries, and in retention of immature forms in the bone marrow, might account in part for altered granulocyte kinetics and function in CML.

THE INTRINSIC defects in chronic myeloid leukemic (CML) cells that lead to hyperleukocytosis, increased immature forms in the blood, and to ultimate hematologic deterioration remain to be defined. Although the chromosomal1 and enzymic2 alterations in CML are well known, their relationships to the functional abnormalities of CML cells are not well understood.

Abnormalities in CML cell surface charge,3 cell deformability,4 nylon-fiber adhesion, and phagocytosis5 have been described and could reflect altered topography, composition, or mobility of membrane glycoproteins.6 The availability of several purified plant and shellfish lectins with defined oligosaccharide specificities7 has permitted better assessment of the structure and function of glycose determinants on membranes of living cells. We have therefore examined the granulocytes from normal, leukocytotic, and chronic myelocytic leukemic patients to study the accessibility of cell surface galactosyl, mannosyl, and sialic acid residues and to analyze their relationship to granulocyte function.

MATERIALS AND METHODS

Separation of Granulocytes

Peripheral blood granulocytes were obtained from patients with Philadelphia chromosome-positive chronic myelogenous leukemia off treatment for at least 2 mo; four patients with benign leukocytosis (diagnoses: pancreatic carcinoma—1 patient; lung carcinoma—1 patient; and acute sepsis—2 patients); and from normal volunteers. Heparinized blood was mixed 10:1 (v:v) with 6% dextrane in saline (Macrodex -2, Pharmacia, Ltd., Piscataway, N.J.) and allowed to sediment for 30 min at 37°C. The supernatant leukocyte-rich plasma was centrifuged at 400 g and resuspended twice in 0.5 ml phosphate-buffered saline, pH 7.2, (PBS). Contaminating RBC were lysed with Tris-1% ammonium chloride. The leukocytes were then layered over a discontinuous gradient of Hypaque-Dextran (bottom layer—1 part 50% sodium diazetrate [Hypaque] in PBS and 2 parts 6% dextran 70; top layer—1 part 30% Hypaque in PBS and 2 parts 6% dextran 70). The cells were centrifuged at 400 g for 12 min, and the cell pellet was harvested, washed twice, and resuspended in PBS. Using this technique, suspensions of mature granulocytes with a purity of greater than 90% were usually obtained, which were then used for all experiments. Suspensions showing less than 80% mature lobulated neutrophils by microscopic observation were discarded.

Nylon-Column Adherence Assay

The technique was adapted from that of MacGregor et al.8 One milliliter of a suspension containing 107 purified peripheral blood granulocytes was passed through a 9-inch Pasteur pipet that had been packed tightly with 50 mg of nylon wool (Associated Biomedic Systems, New York, N.Y.). The columns were then washed with 5.0 ml of PBS and the percentage of cells in the effluent determined by microscopic cell counts. In some experiments, the effluent cells were tested for agglutinability by lectins (vide infra) and compared to the cells in the unfraccionated suspensions.

Lectins

Lectins used included; concanavalin-A (Con-A) (Miles-Yeda Ltd., Rehovoth, Israel) in concentrations of 10–1000 μg/ml; peanut lectin (PNA) (Sigma Chemical, St. Louis, Mo.) 10–2000 μg/ml; and limulin (LIM) (Sigma Chemical) 10–250 μg/ml. The lots of
PNA and Con-A used had been purified by affinity chromatography. Each showed a single homogenous band in polyacrylamide gel electrophoresis. Limulin showed a single major and three minor bands.

**Agglutination Assays**

Aliquots of $2.5 \times 10^5$ purified granulocytes were suspended in 100 µl of PBS containing different concentrations of the lectin, and incubated for 30 min at room temperature in the wells of a microtiter plate (Cooke Engineering Company, Alexandria, Va.). The plates were centrifuged twice at 1000 rpm for 5 mm, the cells gently resuspended by mixing, and replicate samples from each of 3 wells examined microscopically for agglutination. Agglutinates were scored as follows: (−) no agglutination; (+) occasional 2-cell aggregates; (+) frequent 2-cell, occasional 3-cell aggregates; (+ +) many aggregates of 3 or more cells; (+++) macroscopically visible aggregation; (++++) incorporation of the majority of cells present into aggregated clumps.

**Comparative Assays of Binding of I-125 PNA**

Radioactive $^{125}$I-labeled PNA was prepared by a modified chloramine-T method. Samples of $10^6$ cells were incubated in triplicate with 20 µg $^{125}$I-labeled peanut lectin. In order to account for nonspecific uptake of PNA by granulocytes, additional samples were incubated with lectin plus up to 50 mg/ml beta-D-galactose (Sigma Chemical Company). Specific lectin-saccharide binding was defined as cpm bound by cells in the absence of galactose, minus cpm bound in the presence of galactose.

Statistical differences in specific binding between different samples of cells were assessed by analysis of variance.

**Neuraminidase Treatment**

Cells were treated with vibrio cholera neuraminidase (purified protease-free, Behringwerke Pharmaceutical, Nutley, N.J.), 0.1 U/ml for 30 min at 37°C. They were then centrifuged and washed twice before being used in the agglutination assay or the nylon-fiber adherence assay.

**RESULTS**

**Nylon Adherence (Table 1)**

Cells from normal donors, patients with benign leukocytosis (WBC 32–80,000/cu mm), and chronic myelogenous leukemia (WBC 25–600,000/cu mm) were passed through nylon-wool-packed columns, and the percentage of cells in the effluent determined by counting. CML cells showed distinctly decreased adhesiveness; up to 50% of the cells passed through the column. Less than 5% of normal cells usually adhered. The cells of patients with benign leukocytosis due to infection or neoplasm showed normal or increased adherence to nylon wool. Microscopic examination of Wright-stained smears of the effluent cells showed greater than 90% polymorphonuclear leukocytes, as did each unfractionated suspension.

**Agglutination with Peanut Lectin and With Limulin (Table 2)**

Granulocytes from 7 CML patients with leukocyte counts ranging from 16 to 400,000/cu mm, from 4 patients with benign leukocytosis (WBC 32–72,000/cu mm), and from normal donors were examined. Cells from normal donors showed negative to
slight agglutination with peanut lectin at a concentration of 100 μg/ml and slight to moderate agglutination at 1000 μg/ml. Cells from patients with benign leukocytosis showed somewhat more reactivity to peanut lectin. In contrast, cells from patients with chronic myelogenous leukemia showed substantially less agglutination with peanut lectin, even at concentrations of 1000 μg/ml. The cells of three patients (L.W., S.A., B.A.) did not agglutinate at concentrations greater than 2000 μg/ml of PNA. After treatment with vibrio cholera neuraminidase, both normal and CML cells became visibly (greater than 3 +) agglutinable by 50 μg/ml of PNA (not shown in table). Agglutination with limulin at a concentration of 200 μg/ml was readily detectable with normal or leukocytotic cells. The cells of several CML patients showed greater than normal reactivity to 200 μg/ml limulin with macroscopically visible aggregation of cells.

**Binding of Radiolabeled PNA (Table 3)**

Cells from 3 patients with CML bound significantly less 125I-labeled PNA than neutrophils from 5 normal controls. The difference was due to the almost total lack of specific lectin–saccharide binding by CML cells.

**Lectin Reactivity of Nonadherent CML Granulocytes (Table 4)**

Cell suspensions from each of 5 CML patients were passed through nylon-fiber columns. Agglutination by PNA, Con-A, and limulin of the effluent cells was compared with that of their respective unfractioned cell suspensions. PNA (2 mg/ml) agglutinated the unfraccionated CML suspensions weakly, and the effluent cells not at all. Reactivity with Con-A was also decreased in the effluent cell suspensions. On the other hand, reactivity to limulin (100 μg/ml) was increased in 4 of the 5 effluent cell suspensions tested.

**Effects of Neuraminidase (Table 5)**

The cells of 4 normal, 1 leukocytotic, and 3 CML patients were tested for adherence to nylon-fiber columns before and after treatment with 0.1 U/ml of vibrio cholera neuraminidase (VCN). In vitro treatment of granulocytes from CML patients partially reversed their defect in adherence toward normal. VCN treatment had little effect on cells from normal donors or leukocytotic patients.

**DISCUSSION**

It is recognized that CML neutrophils develop from an abnormal clone of stem cells, but the pathogenesis of their massive accumulation in the bone marrow, blood, and spleen has not been explained. It has been

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**Table 4. Lectin Reactivity of Nonadherent CML Granulocytes**

<table>
<thead>
<tr>
<th>Patient</th>
<th>PNA (2 mg/ml)</th>
<th>CON-A (3.2 mg/ml)</th>
<th>LIM (0.1 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR (25,000)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FR, nonadherent</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IA (18,000)</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IA, nonadherent</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SH (100,000)</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SH, nonadherent</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SA (125,000)</td>
<td>±</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>SA, nonadherent</td>
<td>NT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GR (120,000)</td>
<td>±</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>GR, nonadherent</td>
<td>NT</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

±NT, not tested.

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**Table 3. Binding of 125I-Labeled Peanut Lectin by Normal or CML Granulocytes**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Bound/10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR Normal</td>
<td>1,153 ± 50†</td>
<td>886 ± 50</td>
</tr>
<tr>
<td>CO Normal</td>
<td>1,103 ± 73</td>
<td>953 ± 62</td>
</tr>
<tr>
<td>SL Normal</td>
<td>784 ± 106</td>
<td>504 ± 39</td>
</tr>
<tr>
<td>JA Normal</td>
<td>1,033 ± 102</td>
<td>874 ± 40</td>
</tr>
<tr>
<td>WI Normal</td>
<td>1,166 ± 187</td>
<td>860 ± 23</td>
</tr>
<tr>
<td>DL CML</td>
<td>593 ± 70†</td>
<td>532 ± 77</td>
</tr>
<tr>
<td>JW CML</td>
<td>553 ± 43†</td>
<td>515 ± 24</td>
</tr>
<tr>
<td>VJ CML</td>
<td>636 ± 29†</td>
<td>596 ± 75</td>
</tr>
</tbody>
</table>

*Incubated with 20 μg radiolabeled PNA in 100-μl assay volume (see text).
†Mean ± SD of triplicate determinations.
§Significantly different (p < 0.05) from binding by normal granulocytes (one-way analysis of variance).
‡Significantly different (p < 0.01) from binding by normal granulocytes.

**Table 5. Effects of Neuraminidase (VCN) on Granulocyte Nylon-Fiber Adherence**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Untreated</th>
<th>VCN-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA Normal</td>
<td>1.8</td>
<td>1.2†</td>
<td></td>
</tr>
<tr>
<td>XY Normal</td>
<td>0.6†</td>
<td>0.6†</td>
<td></td>
</tr>
<tr>
<td>MA Normal</td>
<td>1.7</td>
<td>6.9†</td>
<td></td>
</tr>
<tr>
<td>GR Normal</td>
<td>2.0</td>
<td>1.2†</td>
<td></td>
</tr>
<tr>
<td>CK Leukocytosis (37,000)</td>
<td>1.8</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>LW CML (600,000)</td>
<td>20.0</td>
<td>13.8‡</td>
<td></td>
</tr>
<tr>
<td>CA CML (120,000)</td>
<td>32.2</td>
<td>22.0‡</td>
<td></td>
</tr>
<tr>
<td>SH CML (100,000)</td>
<td>63.0</td>
<td>51.4‡</td>
<td></td>
</tr>
<tr>
<td>BA CML (68,000)</td>
<td>17.2</td>
<td>11.9‡</td>
<td></td>
</tr>
</tbody>
</table>

*Previously untreated.
†Not significantly different from untreated cells.
‡Significantly different from untreated cells (p < 0.01, paired t test).
suggested that in addition to hyperplasia of committed stem cells and early myeloid forms, the leukocytosis is partly due to the protracted survival of mature and immature neutrophils in the blood, a possible consequence of their curtailed emigration into the tissues or into inflammatory exudates. The data in Table 1 indicate that morphologically mature neutrophils from patients with leukocytotic myeloid leukemia are defective in their ability to adhere to column-packed nylon-wool fibers, an in vitro test that closely reflects in vivo neutrophil margination and migration into inflammatory sites. In contrast, cells from patients with benign leukocytosis showed greater than normal adherence, which may reflect the rapid extravasation of these cells in vivo.

Cell surface complex carbohydrates are thought to be important in regulating intercellular adhesion. The availability of purified lectins, such as peanut lectin (PNA), concanavalin A (Con-A), and limulin (LIM), has aided in the study of oligosaccharide determinants at the outer cell border. PNA binds to cell surface galactosyl sites exposed after removal of sialic acid by neuraminidase treatment. Con-A binds to mannosyl determinants on either neuraminidase-treated or untreated cells, and LIM shows specificity for cell surface sialic acid residues.

Our agglutination studies with neutrophils (Table 2) denote two types of membrane oligosaccharide alterations in CML cells that may be related to their defective nylon-column adherence. First, peripheral blood granulocytes from patients with leukocytotic chronic myelogenous leukemia show reduced agglutination by PNA when compared to normal neutrophils. This is in contrast to the cells of patients with benign leukocytosis, where agglutination by PNA was greater than normal. Second, the cells of several CML patients showed increased agglutination with LIM, denoting increased density or altered distribution of cell surface sialic acid.

The data in Table 3 suggest that reduced agglutination of CML cells by PNA is due to a marked reduction in available binding sites for radioactive lectin. CML cells do not lack membrane galactosyl residues, since both normal and CML cells became highly sensitive to PNA agglutination after neuraminidase treatment.

These cell surface saccharide changes were not present to the same degree in all CML neutrophils. The subpopulation of less adhesive cells isolated by filtration through nylon wool differed from the unfractionated suspension in showing reduced galactosyl (PNA) and mannosyl (Con-A) binding sites, and increased agglutination by LIM.

It is possible that in CML, sialic acid or hypersialylated glycoproteins may mask cell surface receptors that mediate granulocyte adhesion. Whether these alterations reflect the synthesis of a unique glycoprotein by CML cells, a relative increase in normal sialylated glycoproteins, or synthesis of glycoproteins containing more sialyl groups per polypeptide chain cannot be determined from our data. Increased levels of sialyltransferase as well as other glycosyltransferases have been reported in myeloid and lymphoid leukemia. Also, although we (Taub, R. N., Madyastha, K. R., unpublished data) and others have found similar total quantities of neuraminidase-sensitive sialic acid in normal or CML granulocytes, some CML cells have been shown to contain increased amounts of heavily sialylated, possibly unique, glycopeptides in pronase-digested membrane trypsinites. Our data do not exclude the possibility that CML cells lack one or more specific surface glycoproteins bearing receptors for PNA and Con-A. However, this is less likely because of our observation that neuraminidase treatment partially reverses the abnormality in nylon-wool adherence of CML cells (Table 5).

Abnormally sialylated surface glycopeptides have been described in other cells exhibiting malignant behavior, where they may abet the insulation of such cells from exogenous regulatory stimuli or immune interactions. Thus, several studies have demonstrated masking of immunogenic sites on murine leukemia cells by neuraminidase-sensitive determinants. The study of CML cells with their pathognomonic chromosomal marker, their unambiguous morphology, and readily assessable function in vitro should provide an important opportunity to determine the value of this model.

REFERENCES

Masking of neutrophil surface lectin-binding sites in chronic myelogenous leukemia (CML)

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