Antigenic Analysis of Hematopoiesis: II. Expression of Human Neutrophil Antigens on Normal and Leukemic Marrow Cells

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The binding of five antineutrophil monoclonal antibodies, AHN-1, -2, -3, -7, and -8, to normal and leukemic bone marrow cells was studied. AHN-7 bound to many granulocyte precursors, particularly myelocytes, and both lymphoid and blast cells in normal marrow, and to most but not all granulocyte-monocyte progenitors (CFC-GM). AHN-8 bound only to late (band and segmented) neutrophilic cells and not to CFC-GM. AHN-1, -2, -3 bound to morphologically identifiable neutrophil precursors, but not to (day-14) CFC-GM. Approximately half of nonlymphoid leukemia specimens were positive with AHN-1 and AHN-7; conversely, lymphoid leukemia specimens were rarely positive. AHN-1, -2, and -3 were rarely found on leukemia cells. These antineutrophil antibodies appear to detect distinct granulopoietic subgroups and may be useful in the analysis of hematopoietic differentiation and the subclassification of leukemias.

Hybridoma-derived monoclonal antibodies (MoAb) specifically reactive with lymphocyte cell surface molecules have been of great value in the analysis of lymphocyte differentiation and lymphoid neoplasms. MoAb reactive with human neutrophils have been developed and are potentially important tools for the study of granulocytic function. Leukemic cell origins, and granulopoiesis. Antibodies against the My-1 human granulocyte antigen react with morphologically identifiable neutrophil precursors, but not with colony-forming cells of the granulocyte-monocyte lineage (CFC-GM). We have studied five additional antineutrophil monoclonal antibodies for reactivity with human leukemic and normal marrow cells, including CFC-GM. The AHN-7 MoAb reacts with a neutrophil surface protein of 45,000/44,000 apparent molecular weight and binds to peripheral blood basophils, eosinophils, and monocytes, as well as neutrophils (and unpublished data). AHN-8 recognizes a glycoprotein antigen and reacts solely with mature neutrophils in peripheral blood. AHN-1, -2, and -3 bind to a carbohydrate sequence found on several membrane glycolipids and cell surface proteins of neutrophils.

Materials and Methods

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THANK YOU.
Marine monoclonal antibodies AHN-1, -2, -3, -7, -8 were prepared as previously described.12 AHN-1, -2, and -3 are IgM(n) MoAbs; AHN-7 and -8 are IgG1 MoAbs; next spent hybridoma culture supernatant was used as the antibody source for AHN-1, -7, and -3. Nonadherent human bone marrow leukocytes (5 x 10^6) in RPMI 1640 containing 10% (13.600g./ml) diluted acetone 1.0384 L 0.02% protein (Sigma, St. Louis, MO) were resuspended with an equal volume of sterile centrifuged bone marrow. Fab antibodies were neutralized with an equal volume of sterile, centrifuged bone marrow. Low-density nonadherent human bone marrow leukocytes were separated by immune adherence ("panning") into antigen-positive (bound) and antigen-negative (unbound) populations. Aliquots of the panned cell populations were incubated (again) with AHN-7-positive, compared with the negative control, and assayed for growth as described.2 Growth in 2 experiments indicated that the positive control MoAb 28/43/6, over"
90% of the marrow leukocytes and all of the CFC-GM were bound. The AHN-7-positive (bound) population contained 43% of the starting CFC-GM (19%-43% in 4 experiments), suggesting that most, but not all, CFC-GM in normal marrow express the antigen detected by AHN-7. This was not due to nonspecific binding of CFC-GM, as no CFC-GM was detected in the small cell populations bound after negative control antibody treatment (n = 8 experiments). Marrow leukocytes isolated by treatment with AHN-8 MoAb and panning were predominantly (>80% in all 6 experiments) late neutrophilic forms: 22% metamyelocytes, 32% band, and 40% segmented polymorphonuclear leukocytes in the experiment shown (Table 1B). In 6 replicate panning experiments with AHN-8, average cell recovery in the antigen-positive population was 12% (range 7%-14%) of the starting cells. As expected, when these morphologically well differentiated AHN-8-positive cellswere cultured (Table 2B), CFC-GM were absent, and CFC-GM were recovered quantitatively in the antigen-negative fraction (recovery 86% ± 18%, n = 6 experiments). The results indicate that expression of the AHN-8 antigen by hematopoietic cells is confined to the most morphologically mature cells in the neutrophilic series. The bound population of marrow leukocytes after treatment with MoAb AHN-1, -2, or -3, contained approximately 75% morphologically identifiable neutrophil precursors, including progranulocytes, plus rare myeloid blasts. The unbound population contained predominantly erythroid and lymphoid cells, as well as small numbers of eosinophils, basophils, and megakaryocytes (Table 1C). results for AHN-2 and AHN-3 were essentially identical to those for AHN-1 and are omitted from Table 1C. When the panned populations were cultured, CFC-GM were recovered quantitatively in the antigen-negative fractions and were absent from antigen-positive fractions (Table 2C). The IgM MoAb AHN-1, -2, and -3 were also tested for cellular reactivity by complement-mediated cytolysis. These MoAb were strongly cytotoxic to HL-60 target cells, as measured either by trypan blue dye exclusion or by colony-formation assay (data not shown). Viable marrow cells (isolated by density-gradient centrifugation) remaining after cytolysis with AHN-1, -2, or -3 antibody plus complement were greatly enriched for erythroid, lymphoid, and blast cells (Table 1D); essentially all morphologically identified neutrophil precursors were removed. However, no reduction of CFC-GM numbers was observed, either in individual experiments or in pooled data from multiple experiments (Table 3A). AHN-2 was tested as diluted (1:50) ascites fluid; the slight observed effect of AHN-2 on CFC-GM numbers may be due to unknown substances in the ascites fluid. In contrast, CFC-GM were almost completely eliminated by positive control MoAb 28/43/6 plus complement under these conditions. Marrow cells surviving initial treat-
ment with AHN antibody plus complement were treated a second time with fresh antibody plus complement; no incremental effect on CFC-GM number was observed (Table 3B), confirming that initial conditions included excess antibody and complement. Antibody excess was demonstrated directly by preincubation of MoAb with marrow leukocytes prior to use in anti-HL-60 cell cytolysis experiments: marrow-preabsorbed antibody was as strong cytolytic for HL-60 cells as unabsorbed antibody (data not shown). These results suggest that the antigen(s) identified by AHN-1, -2, and -3 are expressed by morphologically identifiable neutrophilic precursors, but not by cells of other lineages nor by day-14 CFC-GM.

Leukemic blast cell specimens were analyzed by indirect immunofluorescence (as previously described) for expression of these AHN MoAb. Only specimens containing ≥80% leukemic cells were analyzed. A finding of ≥20% fluorescent cells (above background with isotype-matched control MoAb) indicated expression of an antigen by the leukemic cells and was defined as a positive specimen. MoAb AHN-1 and AHN-7 reacted with approximately 50% (55% and 49%, respectively) of the specimens from patients with morphologically defined acute nonlymphoblastic leukemia (Table 4). A single infant with CALLA-negative acute lymphoid leukemia (ALL) was positive for AHN-1. Binding of certain antineutrophil MoAb to rare ALL blast cell specimens has been previously noted. AHN-8 reacted with only 1 of 34 ANLL and no ALL patients tested; the sole AHN-8-positive patient was only marginally positive (22% fluorescent cells) and was AHN-8-negative at relapse.

DISCUSSION

It appears that AHN-7, which identifies an antigen on neutrophils, eosinophils, basophils, and monocytes in peripheral blood, identifies the precursors of these cells and also a subset of mononuclear cells, including blast cells in normal marrow. Cells at the myelocyte stage were most uniformly positive for AHN-7 in panning experiments, suggesting that antigen expression is maximal at this point in development. The observation that only 54% of the CFC-GM "missing" from the unbound fraction was detected in the bound fraction can be attributed, at least in part, to loss of viable cells. The recovery of bound cells required vigorous pipetting; a mechanically induced reduction of colony-forming efficiency might therefore be expected. In the experiment shown, 2 ml of neat AHN-7 supernatant was used, fourfold excess volume over that used in usual experiments, to assure MoAb excess. Partition of antigen-positive from antigen-negative cells was demonstrated by immunofluorescence assay, but some weakly positive cells were still unbound. The purity of the antigen-negative fraction may depend on the physical method used to collect unbound cells. Conversely, the purity of the bound fraction may depend on the presence of Fe-receptorbearing cells in the suspension. As no CFC-GM were unbound after treatment with positive control MoAb
263 28/43/6, however, and no nonspecific binding of CFC-
264 GM was observed, these experiments suggest that
265 CFC-GM in normal marrow are at least quantitatively
266 heterogeneous for expression of the antigen identified
267 by AHN-7. An alternative hypothesis, which we con-
268 sider less likely, is that a helper cell population19 was
269 partitioned from the CFC-GM by the procedure. Fur-
270 thermore, we suggest that the AHN-7-positive popu-
271 lation does not correspond to a single morphologically
272 defined cell category, but may relate to the prolifer-
273 ative state as has been shown for the My-7 antigen.19
274 The AHN-7 antigens may prove to be important in the
275 further analysis of hematopoiesis.
276 The antigen identified by AHN-8 is expressed
277 strongly only very late in neutrophilic maturation and
278 might be important in surface-dependent mature neu-
279 trophil function. The MoAb AHN-1, -2, and -3 iden-
280 tify a cell population very similar to that defined by the
281 glycolipid2 My-1 differentiation antigen of human
282 neutrophils. Normal cells of lineages other than neu-
283 trophile were not identified by AHN-1, -2, or -3, in
284 contrast to AHN-7. Immature neutrophils were AHN-
285 1-positive (but AHN-8-negative), but CFC-GM were
286 AHN-1-negative. The results of cell separation using
287 AHN-1, -2, -3 and panning were confirmed using
288 complement-mediated cytotoxicity. The removal of
289 antibody-positive cells was more complete using
290 complement, but the results of CFC-GM assays were
291 identical, showing high CFC-GM recovery in both
292 antibody-negative populations. Another laboratory17
293 has observed that CFC-GM express AHN-1, -2, -3
294 antigens. Possible explanations for these contrasting
295 results are that the other studies used a different
296 source of colony-stimulating factor (leukocyte condi-
297 tioned media), a different preparation of MoAb (as-
298 cites), and a different duration of culture for CFC-GM
299 (8 days).
300 In the analysis of ANLL blast cell specimens with
301 antmyeloid MoAb, normal granulopoietic cells
302 expressing the detected antigens might contaminate
303 the leukemic cells. False positive results arising in this
304 way were excluded by the requirement that ≥20%
305 (above background) of cells be fluorescent in a sample
306 containing ≥80% leukemic cells. Thus, the percentages
307 of positive specimens shown (Table 4) are minimum
308 estimates. As many ANLL specimens reacted with
309 AHN-1 and AHN-7, these antibodies might, when
310 positive, be helpful in the distiction of ANLL from
311 ALL. It is intriguing that ANLL blast cells rarely (if
312 ever) bind AHN-8. Conceivably, AHN-8-positive cells
313 may not be susceptible to leukemic transformation, or,
314 once transformed, the leukemic cells may obligately
315 lose this antigen. Alternatively, nonlymphocytic leu-
316 kemic cells may be unable to differentiate to the stage
317 of AHN-8 antigen expression.19 Whatever the mecha-
318 nism, the rarity of AHN-8-positive ANLL blast cells is
319 analogous to the expression of surface antigens (e.g.,
320 T3, surface immunoglobulin) of normal mature lymph-
321 oid cells only on rare ALL blast cells.17
322 We have shown that the MoAb AHN-1, -2, and -3
323 detect a lineage- and stage-specific neutrophil differ-
entiation antigen very similar to the My-1 antigen\textsuperscript{3} not present on day-14 CFC-GM. The AHN-7 MoAb detects an antigen expressed by maturing granulocytic cells, by many lymphoid and blast cells, and by many, but not all, CFC-GM. The AHN-8 MoAb detects an antigen expressed very late in neutrophil differentiation, first on metamyelocytes. As these AHN antibodies define distinct, but overlapping, sets of granulopoietic cells, they are of potential use in the study of the mechanisms of normal cellular differentiation and the aberrant differentiation processes in leukemia.

ACKNOWLEDGMENT

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REFERENCES

14. (4) Huang LC, Civin CI, Magee JS, Shaper JH, Giebult V: MY7, the human myeloid-specific antigen detected by mouse monoclonal antibodies, is a major sequence found in leuka-N-sequences.
### Table 1. Differential Counts of AHR-Transplanted Marrow Cell Specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Male (%)</th>
<th>Female (%)</th>
<th>Total (%)</th>
<th>Progenitors (%)</th>
<th>Monocytes (%)</th>
<th>Myeloid (%)</th>
<th>Macrophages (%)</th>
<th>Neutrophils (%)</th>
<th>Monocytes (%)</th>
<th>Neutrophils (%)</th>
<th>Macrophages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>11</td>
<td>9</td>
<td>20</td>
<td>35</td>
<td>26</td>
<td>15</td>
<td>10</td>
<td>76</td>
<td>6</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Paternal</td>
<td>14</td>
<td>13</td>
<td>27</td>
<td>38</td>
<td>22</td>
<td>16</td>
<td>9</td>
<td>74</td>
<td>8</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 2. Separation By "Passing" of AHR Antibody-Treated Marrow Cells: CFC-GM and Positive and Negative Fractions

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CFC-GM Recovery (%)</th>
<th>CFC-GM/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>88</td>
<td>23 (6)</td>
</tr>
<tr>
<td>Paternal</td>
<td>92</td>
<td>28 (7)</td>
</tr>
</tbody>
</table>

**Values** represent 100% x (Viability cell number in fraction - initial cell number) after treatment with antibody and preplating procedure.

**Mean** (standard deviation) of triplicates determined, rounded to nearest integer.

**Product** of CFC-GM/10^6 cells x number viable cells in fraction.

**CFC-GM/10^6 cells** used as input for experiments.
Table 3

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Visible Cells</th>
<th>Colony Count</th>
<th>Percent of Colonies</th>
<th>Percent of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant 1</td>
<td>Resistant 2</td>
<td>Single Ex 1</td>
<td>Positive Ex 2</td>
</tr>
<tr>
<td>TEPC 183</td>
<td>96 (8)</td>
<td>82 (4)</td>
<td>100 (10)</td>
<td>100 (10)</td>
</tr>
<tr>
<td>AHN-1</td>
<td>41 (6)</td>
<td>81 (8)</td>
<td>83 (6)</td>
<td>83 (6)</td>
</tr>
<tr>
<td>AHN-2</td>
<td>50 (14)</td>
<td>40 (4)</td>
<td>79 (18)</td>
<td>79 (18)</td>
</tr>
<tr>
<td>AHN-3</td>
<td>72 (4)</td>
<td>60 (5)</td>
<td>84 (17)</td>
<td>84 (17)</td>
</tr>
<tr>
<td>28/43/8</td>
<td>15 (3)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>Antibody</th>
<th>N/A</th>
<th>N/A</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEPC 183</td>
<td>114 (6)</td>
<td>114 (6)</td>
<td>100</td>
</tr>
<tr>
<td>AHN-1</td>
<td>143 (21)</td>
<td>143 (21)</td>
<td>100</td>
</tr>
<tr>
<td>AHN-2</td>
<td>86 (9)</td>
<td>86 (9)</td>
<td>100</td>
</tr>
<tr>
<td>AHN-3</td>
<td>101 (5)</td>
<td>101 (5)</td>
<td>100</td>
</tr>
<tr>
<td>28/43/8</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent number of 100% x (Visible cells recovered - initial cell number). Mean (standard deviation) for four experiments is shown.

**Representative experiment with marrow from a normal donor. Mean (standard deviation) of replicates cultured of 107 cells is shown.

*Values represent mean (SEM) of colony counts from different experiments (n = 11) expressed as percent of negative control (TEPC) 183 in the same experiment.

*Antibody used as divided 1:500 assess fluid.

Table 5

<table>
<thead>
<tr>
<th>Disease</th>
<th>AHN-1</th>
<th>AHN-2</th>
<th>AHN-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute nonlymphoblastic leukemia</td>
<td>55%</td>
<td>48%</td>
<td>3%</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>(23/42)</td>
<td>(18/36)</td>
<td>(1/24)</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>CALL-positive</td>
<td>(0/3)</td>
<td>(0/2)</td>
<td>(0/2)</td>
</tr>
<tr>
<td>CALL-negative</td>
<td>33%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>HLA-DR-positive</td>
<td>(1/2)</td>
<td>(0/2)</td>
<td>(0/2)</td>
</tr>
<tr>
<td>T cell</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>412</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Chronic phase</td>
<td>(1/1)</td>
<td>(0/1)</td>
<td>(0/1)</td>
</tr>
<tr>
<td>Blastic crisis</td>
<td>100%</td>
<td>50%</td>
<td>0%</td>
</tr>
<tr>
<td>412</td>
<td>100%</td>
<td>50%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Acute nonlymphoblastic leukemia was defined morphologically.

*Acute lymphoblastic leukemia was defined morphologically and by expression of CALLA, T and B lymphoid and HLA-DR surface markers (direct immunofluorescence). T cell Leu-1 or T11 positivity was used to define T lymphoid leukemia. Chronic myeloid leukemia was defined clinically and by morphology.

*Percent positive specimens (number positive/number tested).

*Defined as a 20% fluorescent cells above age-matched control background fluorescence.