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

Lewis C. Strauss, Keith M. Skubitz, J. Thomas August, and Curt I. Civin

024 The binding of five antineutrophil monoclonal antibodies,
025 AHN-1, -2, -3, -7, and -8, to normal and leukemic bone
026 marrow cells was studied. AHN-7 bound to many granulocyte
027 precursors, particularly myelocytes, and both lymphoid
028 and blast cells in normal marrow, and to most but not
029 all granulocyte-macrophage progenitors (CFC-GM). AHN-8
030 bound only to late (band and segmented) neutrophilic cells
031 and not to CFC-GM. AHN-1, -2, -3 bound to morphologically
032 identifiable neutrophil precursors, but not to (day-14) CFC-
033 GM. Approximately half of nonlymphoid leukemia specimens
034 were positive with AHN-1 or AHN-7; by contrast,
035 lymphoid leukemia specimens were rarely positive. AHN-8
036 was rarely found on leukemic cells. These antineutrophil
037 antibodies appear to detect distinct granulopoietic subsets
038 and may be useful in the analysis of hematologic differentiation
039 and in the subclassification of leukemias.

Notes: we would prefer
MeAb as an abbreviation
for monoclonal antibody
(throughout the paper).

Reprints (14)

040 **HYBRIDOMA-DERIVED** monoclonal antibodies
041 (MoAb) specifically reactive with lymphocyte
042 cell surface molecules have been of great value in the
043 analysis of lymphocyte differentiation and lymphoid
044 neoplasia. MoAb reactive with human neutrophils
045 have been developed¹ and are potentially important
046 tools for the study of granulocyte function,² leukemic
047 cell origins,³ and granulopoiesis.⁴ Antibodies against
048 the My-1 human granulocyte antigen react with morphologically
049 identifiable neutrophil precursors, but not
050 with colony-forming cells of the granulocyte-monocyte
051 lineage (CFC-GM).⁵ We have studied five additional
052 antineutrophil monoclonal antibodies for reactivity
053 with human leukemic and normal marrow cells,
054 including CFC-GM. The AHN-7 MoAb reacts with a
055 neutrophil surface protein of 45,000/65,000 apparent
056 molecular weight and binds to peripheral blood basophils,
057 eosinophils, and monocytes, as well as neutrophils⁶ (and unpublished data). AHN-8 recognizes a
058 glycolipid antigen and reacts solely with mature neutrophils
059 in peripheral blood.⁷ AHN-1, -2, and -3 bind to a carbohydrate
060 sequence found on several membrane glycolipids and
061 proteins of neutrophils.^{7,8}

Neutrophil Antigens
on Marrow Cells

28,000 - 65,000

as well as glycolipids,

URGENT

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MATERIALS AND METHODS

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065 Blood—7360-203-4 swai 12-14-83

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067 Murine monoclonal antibodies AHN-1, -2, -3, -7, -8 were pre-
068 pared as previously described.^{2a} AHN-1, -2, and -3 are IgM(κ)
069 MoAb; AHN-7 and -8 are IgG1(κ) MoAb. Neat spent hybridoma
070 culture supernatant was used as the antibody source for AHN-1, -3,
071 -7, -8; diluted ascites fluid (1:50) was used for AHN-2. Negative
072 controls were the IgM myeloma protein, TEPC 183, used as diluted
073 ascites fluid (1:50), and MOPC 21, a IgG1 myeloma protein, used as
074 diluted ascites (1:50) or neat spent supernatant of the P3X63.AG8
075 cell line (American Type Culture Collection, Rockville, MD). These
076 isotype-matched myeloma proteins react with no known antigens
077 and were utilized in parallel with AHN MoAbs to control for the
078 possible binding of MoAb due to the Fc rather than the Fab region.
079 The IgG2b MoAb, 28/43/6, which binds to lymphocytes from all
080 donors tested,² was used as a positive control. All antibodies were
081 used in greater than eightfold excess.

082 Blood cells and bone marrow cells were prepared, and indirect
083 immunofluorescence assays were performed as previously
084 described.⁸ Background fluorescence obtained with negative control
085 antibodies was <5% and was subtracted from that obtained with
086 AHN MoAb. Low-density nonadherent human bone marrow leuko-
087 cytes [5×10^5 /ml in RPMI 1640 (Flow, Rockville, MD) containing
088 0.2% bovine serum albumin (BSA; Sigma, St. Louis, MO)] were
089 routinely incubated with an equal volume of sterile, centrifuged
090 (15,600 g, 15 min, 4°C) MoAb for 20 min (22°C). The cells were
091 washed twice, then either (A) "panned" on a goat anti-mouse
092 immunoglobulin-coated Petri dish, using the previously described
093 immune adherence "panning" technique,^{8,9} or (B) resuspended in
094 RPMI 1640 containing 0.2% BSA, DNase I (250 Kunitz U/ml;
095 Sigma, St. Louis, MO) and rabbit complement (Cedarlane "Low-
096 Tox H," Accurate Chemical Corp., Westbury, NY) at 1:8 dilution,
097 as described.⁸ Cells recovered from these procedures were counted
098 (viable cell count by trypan blue dye exclusion), examined for
099 morphology, and placed in semisolid agar tissue culture medium
100 (containing 5% v/v human placenta conditioned medium) for
101 growth and enumeration of CFC-GM.¹⁰

102 RESULTS

103 Normal human marrow leukocytes were examined
104 for reactivity with the AHN-7 antibody. Low-density
105 nonadherent marrow leukocytes were separated by
106 immune adherence ("panning") into antigen-positive
107 (bound) and antigen-negative (unbound) populations.
108 Aliquots of the panned cell populations were incubated
109 (again) with AHN-7 antibody, then analyzed by indi-
110 rect immunofluorescence for expression of AHN-7
111 antigen: >90% of the cells in the bound fraction were
112 AHN-7-positive, compared with 35% in the unsepa-
113 rated population; marked variation in fluorescence
114 staining intensity was observed. Approximately 10% of
115 the cells in the unbound fraction were (weakly) AHN-
116 7-positive.

117 Fractions obtained after treatment with excess
118 AHN-7 and panning were examined morphologically
119 and assayed for CFC-GM. The AHN-7-positive
120 (bound) marrow cell fraction (Table 1A) contained
121 morphologically identifiable eosinophil, basophil, and
122 neutrophil precursor cells, including myeloblasts, and
123 was particularly rich in neutrophilic myelocytes (32%
124 and 31% in 2 experiments). A large number of lym-
125 phoid cells and blast cells of several lineages were also
126 seen in the bound fraction. Thus, most or all myelo-
127 cytes and subsets of these other marrow cell types
128 express the antigen identified by AHN-7. The AHN-
129 7-negative (unbound) population was depleted of
130 CFC-GM (Table 2A): only 21% (21%-38% in 3
131 experiments) of control CFC-GM remained. In con-
132 trast, with the positive control MoAb 28/43/6, over



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136 90% of the marrow leukocytes and all of the CFC-GM
137 were bound.⁴ The AHN-7-positive (bound) population
138 contained 43% of the starting CFC-GM (19%–43% in
139 4 experiments), suggesting that most, but not all,
140 CFC-GM in normal marrow express the antigen
141 detected by AHN-7. This was not due to nonspecific
142 binding of CFC-GM, as no CFC-GM ~~was~~ ^{were} ever
143 detected in the small cell populations bound after
144 negative control antibody treatment ($n = 8$ experi-
145 ments).

146 Marrow leukocytes isolated by treatment with
147 AHN-8 MoAb and panning were predominantly
148 (>80% in all 6 experiments) late neutrophilic forms:
149 22% metamyelocytes, 32% band, and 40% segmented
150 polymorphonuclear leukocytes in the experiment
151 shown (Table 1B). In 6 replicate panning experiments
152 with AHN-8, average cell recovery in the antigen-
153 positive population was 12% (range 7%–14%) of the
154 starting cells. As expected, when these morphologi-
155 cally well differentiated AHN-8-positive cells were
156 cultured (Table 2B), CFC-GM were absent, and CFC-
157 GM were recovered quantitatively in the antigen-
158 negative fraction (recovery $86\% \pm 18\%$, $n = 6$ experi-
159 ments). The results indicate that expression of the
160 AHN-8 antigen by hematopoietic cells is confined to
161 the most morphologically mature cells in the neutro-
162 philic series.

163 The bound population of marrow leukocytes after
164 treatment with MoAb AHN-1, -2, or -3, contained
165 approximately 75% morphologically identifiable neu-
166 trophil precursors, including progranulocytes, plus
167 rare myeloid blasts. The unbound population con-
168 tained predominantly erythroid and lymphoid cells, as
169 well as small numbers of eosinophils, basophils, and
170 megakaryocytes (Table 1C; results for AHN-2 and
171 AHN-3 were essentially identical to those for AHN-1
172 and are omitted from Table 1, ~~Table B~~). When the
173 panned populations were cultured, CFC-GM were
174 recovered quantitatively in the antigen-negative frac-
175 tions and were absent from antigen-positive fractions
176 (Table 2C).

177 The IgM MoAb AHN-1, -2, and -3 were also tested
178 for cellular reactivity by complement-mediated cytoly-
179 sis. These MoAb were strongly cytotoxic to HL-60
180 target cells, as measured either by trypan blue dye
181 exclusion⁵ or by colony-formation assay (data not
182 shown). Viable marrow cells (isolated by density-
183 gradient centrifugation) remaining after cytotoxicity with
184 AHN-1, -2, or -3 antibody plus complement were
185 greatly enriched for erythroid, lymphoid, and blast
186 cells (Table 1D); essentially all morphologically identi-
187 fied neutrophil precursors were removed. However, no
188 reduction of CFC-GM numbers was observed, either
189 in individual experiments or in pooled data from
190 multiple experiments (Table 3A). AHN-2 was tested
191 as diluted (1:50) ascites fluid; the slight observed effect
192 of AHN-2 on CFC-GM numbers may be due to
193 unknown substances in the ascites fluid. In contrast,
194 CFC-GM were almost completely eliminated by posi-
195 tive control MoAb 28/43/6 plus complement under
196 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 cytotoxicity experiments: marrow-preabsorbed antibody was as strongly cytotoxic 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 described⁶) for expression of these AHN MoAb. Only specimens containing $\geq 80\%$ leukemic cells were analyzed. A finding of $\geq 20\%$ 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 half (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.¹¹ 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; some 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 Fc-receptor-bearing cells in the suspension. As no CFC-GM were unbound after treatment with positive control MoAb

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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 population¹² was
269 partitioned from the CFC-GM by the procedure. Fur-
270 thermore, we suggest that the AHN-7-positive popula-
271 tion does not correspond to a single morphologically
272 defined cell category, but may relate to the prolifera-
273 tive state as has been shown for the My-7 antigen.¹³
274 The AHN-7 antigen 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 identi-
280 fy a cell population very similar to that defined by the
281 glycolipid¹⁴ My-1 differentiation antigen of human
282 neutrophils. Normal cells of lineages other than neu-
283 trophilic 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 laboratory⁷
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 antimyeloid 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 $\geq 20\%$
305 (above background) of cells be fluorescent in a sample
306 containing $\geq 80\%$ 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 distinction 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.¹⁵ 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 lym-
321 phoid cells only on rare ALL blast cells.¹

322 We have shown that the MoAb AHN-1, -2, and -3
323 detect a lineage- and stage-specific neutrophil differ-

have "false background"
to after "fluorescent"

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327 entiation antigen very similar to the My-1 antigen⁴ not
328 present on day-14 CFC-GM. The AHN-7 MoAb
329 detects an antigen expressed by maturing granulocytic
330 cells, by many lymphoid and blast cells, and by many,
331 but not all, CFC-GM. The AHN-8 MoAb detects an
332 antigen expressed very late in neutrophil differentia-
333 tion, first on metamyelocytes. As these AHN antibod-
334 ies define distinct, but overlapping, sets of granulo-
335 poietic cells, they are of potential use in the study of the
336 mechanisms of normal cellular differentiation and the
337 aberrant differentiation processes in leukemia.

338

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Table 1. Differential Counts of AHN-Treated Marrow Cell Specimens*

	Blat (%)	Progranulocyte (%)	Myelocyte (%)	Metap. + Band + Polyt (%)	Lymphoid (%)	Erythroid (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)
(A) AHN-7 treated									
Exp. 1									
MOPC 21—unbound	11	6	18	33	21	8	8	0.5	0.5
AHN-7—unbound	7	6	3	36	26	19	4	0	0
AHN-7—bound	10	9	32	18	29	0	2	1	1
Exp. 2									
MOPC 21—unbound	12	10	18	12	18	26	2	0.5	0.5
AHN-7—unbound	13	10	6	6	17	44	4	0	0
AHN-8—bound	16	4	31	10	36	1	1	0.5	1
(B) AHN-8 treated									
MOPC 21—unbound	6	6	18	51	18	1	0	0	0
AHN-8—bound	0	0	1	94	5	0	0	0	0
(C) AHN-1 treated									
TEPC 183—unbound	6	13	7	16	36	21	2	0.5	0.5
AHN-1—unbound	13	3	3	4	37	36	4	0.5	0.5
AHN-1—bound	2	20	12	42	19	3	1	0	0
(D) AHN-1 cytostatic									
TEPC 183—cytostatic	6	14	16	14	43	8	1	0	0
AHN-1—cytostatic	6	0	0	0	76	13	2	0	0

*Percent of cells based on 500-2,000 cell differential counts (rounded) of Wright-Giemsa-stained smears from a representative experiment (mononuclear cells, band forms, and polymorphonuclear leukocytes).

†Eosinophils and basophils were defined by characteristic secondary granulation; thus, myelocytes and more mature cells in each lineage were counted as eosinophils and basophils, respectively. ‡Results obtained after treatment with AHN-2 or AHN-3 were essentially identical to AHN-1.

Table 2. Separation By "Panning" of AHN Antibody-Treated Marrow Cells: CFC-GM in Positive and Negative Fractions

	Viable Cell Recovery*	CFC-GM/10 ⁵ Cells†	CFC-GM Recovered‡
(A) MOPC 21	88	23 (5)	2,000
(sup§)—unbound			
MOPC 21 (sup)—bound	<1	ND	ND
AHN-7—unbound	52	8 (3)	412
AHN-7—bound	34	25 (3)	860
(B) MOPC 21	84	48 (3)	3,820
(sec§)—unbound			
MOPC 21 (sec)—bound	12	0 (0)	0
AHN-8—unbound	82	48 (2)	3,940
AHN-8—bound	14	1 (0)	20
(C) TEPC 183—unbound	98	77 (10)	8,320
TEPC 183—bound	3	0 (0)	0
AHN-1—unbound	62	136 (2)	9,280
AHN-1—bound	21	<1 (0)	10
AHN-2—unbound	47	172 (8)	8,920
AHN-2—bound	18	2 (1)	30
AHN-3—unbound	62	129 (10)	8,800
AHN-3—bound	16	2 (1)	30

*Values represent 100% × (Viable cell number in fraction ÷ initial cell number) after treatment with antibody and panning procedure.

†Mean (standard deviation) of triplicate determinations, rounded to integer.

‡Product of CFC-GM/10⁵ cells × number viable cells in fraction.

§MOPC 21 (sup), neat supernatant of P3X63.AG8; MOPC 21 (sec), diluted (1:50) ascites fluid.

ND, not done.

Table 3

(A) Treatment of Normal Bone Marrow Cells With AHN-1, 2, 3
Antibody Plus Complement: Effect on CFC-GM

Antibody	Visible Cell Recovery* (%)	Colony Count Single Exp. †	Colony Count Pooled Exp. ‡
TEPC 183	98 (8)	82 (4)	(100) (0)
AHN-1	41 (4)	81 (9)	93 (8)
AHN-2§	50 (14)	40 (4)	79 (9)
AHN-3	72 (4)	60 (5)	94 (7)
28/43/6			
(positive control)	15 (3)	0 (0)	1 (1)

(B) CFC-GM in Residual Marrow Cells After Double-Treatment
With Antibody Plus Complement

Antibody (1)	Antibody (2)	Colony Count Mean (SD)†	Percent of Control
None	None	93 (6)	82
TEPC 183	TEPC 183	114 (5)	[100]
AHN-1	AHN-1	143 (21)	125
AHN-2§	AHN-2§	85 (9)	75
AHN-3	AHN-3	101 (5)	89
TEPC 183	28/43/6	0 (0)	0

*Values represent number of 100% × (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 triplicate cultures of 10⁵ 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 diluted (1:50) ascites fluid.

Table 4. Binding of AHN Antibodies to Leukemic Cell Specimens

Disease*	Percent Positive Specimens†		
	AHN-1	AHN-7	AHN-8
Acute nonlymphoblastic leukemia	55% (23/42)	49% (19/39)	3% (1/34)
Acute lymphocytic leukemia			
CALLA-positive	0% (0/18)	0% (0/9)	0% (0/6)
HLA-DR-positive/CALLA-negative	33% (1/3)	0% (0/3)	0% (0/2)
T cell	0% (0/3)	0% (0/2)	0% (0/2)
Chronic myeloid leukemia			
Chronic phase	100% (1/1)	0% (0/1)	0% (0/1)
Blast crisis (myeloid)	100% (1/1)	50% (1/2)	0% (0/1)

*Acute nonlymphoblastic leukemia was defined morphologically; acute lymphoid leukemia was defined morphologically and by expression of CALLA, T and B lymphoid and HLA-DR surface markers (indirect 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 ≥20% fluorescent cells above isotype-matched control background fluorescence.

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