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Criteria and Classification of Hybrid Acute Leukemia in 72 Acute Leukemias Based Mainly on Flow Cytometric Analysis

Sadao AOKI, Nobuhiko NOMOTO, Souichi MARUYAMA, Shoji SHINADA* and Akira SHIBATA

Department of Internal Medicine I, Niigata University School of Medicine, Asahimachi 1, Niigata 951, Japan, *Blood Transfusion Division, Niigata University Medical Hospital

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Summary. **Phenotypes of leukemic cells can be determined through dual staining with pairs of FITC-labeled and PE-labeled monoclonal antibodies using a laser flow cytometer. Hybrid acute leukemia (HAL) was diagnosed when leukemic cells expressed 2 or more lymphoid markers and at least on myeloid marker simultaneously. Based on this criteria, nineteen out of 72 cases with untreated acute leukemia were diagnosed as HAL, 15 of 29 (51%) patients with acute lymphoblastic leukemia and 4 of 43 (9%) patients with acute non-lymphocytic leukemia were diagnosed as having HAL. We classified these HAL cells into 4 types by the following items. Type I: leukemic cells expressing only CD33 and 2 or more B-cell antigens; Type II: only CD33 and 2 or more T-cell antigens; Type III: two myeloid antigens (CD13, CD33) and 2 or more B-cell antigens; and Type IV: at least 2 of 3 myeloid antigens (CD13, CD33), peroxidase) and 2 or more T-cell antigens. There were 7 Type I cases and 3 Type II cases. These two types were thought to be ALL accompanied with positive CD33 alone, which meant a weak specificity as a myeloid marker. There were 6 Type III cases and 3 Type IV cases. Types III and IV were regarded as definite HAL since they had both myeloid and lymphoid antigens distinctly. The incidence of definite HAL among 72 acute leukemias was 12.5%.**

INTRODUCTION

In recent years, instruments for flow cytometric analyses have been improved in various aspects; especially increments of fluorescence-detecting sensitivity have markedly increased. Flow cytometry (FCM) has become useful not only in the diagnosis of leukemia types but also in the prediction of their prognoses. In our previous study,[1] we described various phenotypes of leukemic cells as determined by FCM.

In accumulating cases with detailed phenotype analyses, surface markers in some leukemia did not correlate with the blood cell differentiation pathway postulated by Foon et al.[2] They were classified in terms of "lineage infidelity," or "unclassified" leukemia.[3,4]

In 1987, Gale proposed the term "hybrid acute leukemia (HAL)" in which leukemic cells expressed two or more lineage markers simultaneously.[5] He designated the terms "biphenotypic leukemia" in which a single leukemic cell expressed multiple lineage markers, and "bilineal leukemia" in which multiple clones of different lineage were present.

We analyzed leukemic cells from 72 patients by dual-staining with various pairs of monoclonal antibodies (MoAb), using a recent model flow cytometer. We found that 19 cases were classified as HAL satisfying Gale's diagnostic criteria.[5] This paper describes our criteria for and classification of HAL.

MATERIALS AND METHODS

Patients

From October 1988 to October 1989, 72 patients with acute leukemia in whom the peripheral blood or bone marrow contained at least 40% blasts and with no previous antileukemic therapy were subjected to this study. The diagnosis, sex and ages of the patients are shown in Table 1.

Surface marker analyses

Staining procedure was the same as that described in previous reports.[1,6] Briefly, pairs of fluorescein (FITC)- and phycoerythrin (PE)- labeled antibodies were added to 0.1 ml of EDTA- heparin-added peripheral

blood or bone marrow. The specimens were kept at 4℃ for 30 min. Then they were washed in phosphate-buffered saline (PBS, Ca^{++}free, pH 7.5) followed by hemolysis and fixation in an FACS lysing solution (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Each stained-cell material was suspended in 0.5 ml PBS.

A FACScan (Beckton and Dickinson, Mountain View, CA, USA) was used as FCM throughout this study. Names of the antibodies and comments were registered in "Simulset" software so that the analytical results might be printed out automatically and immediately after analysis.

Gating of cells was performed as described elsewhere.[1,6] On a cytogram in which right angle scattering rays were plotted against forward scattering rays, contaminants such as debris, erythrocytes and granulocytes were excluded as completely as possible.

Monoclonal antibodies

Pairs of MoAbs labeled with FITC and PE are shown in Table 2. As lymphoid antigens markers, CD3, CD4, CD8, CD19, CD2, CD20, CD10, CD1 and CD21 were used, where CD13 and CD33 were the myeloid antigen markers, CD14 and CD36 indispensable to monocytes, and HLADR, CD7 and CD25 as supplementary markers.

The list of MoAbs and that of antigens for lineage classification are shown in Table 2 and Table 3.

Terminal deoxynucleotidyl transferase (TdT) activity

TdT was tested under a fluorescent microscope by an indirect fluorescent antibody method (Immunotech S. A., Marseille, France) described elsewhere.[1,6]

Peroxidase reaction

Peroxidase staining was carried out according to the method of the International Standardization Organization[7] and samples were observed under a light microscope.

Criteria for hycrid acute leukemia

A diagnosis of HAL was made when 2 or more lymphoid antigens and at least one myeloid antigen were simultaneously positive.

Table 1. Patient Characteristics

Diag.	Male	Female	Total	Age	
				Range	Average
ALL	16	13	29	1-82	38.2
ANLL	29	14	43	22-79	55.5
Total	45	27	72	1-82	48.5

ALL; acute lymphoblastic leukemia, ANLL; acute non-lymphocytic leukemia

Table 2. Pairs of monoclonal antibodies used with FACScan two-color analysis

FITC	PE
CD4 (Leu3 *)	CD8 (Leu2 *)
CD3 (Leu4 *)	CD19 (Leu12 *)
CD2 (Leu5 *)	antiHLA-DR *
CD13 (MCS2 * *)	CD20 (Leu16 *)
CD36 (OKM5 * * *)	CD14 (LeuM3 *)
CD10 (OKBCALLA * * *)	CD33 (MY9 * * * *)
CD1 (OKT6 * * *)	CD21 (OKB7 * * *)
CD7 (Leu9 *)	CD25 (2A3 *)

* ; Becton Dickinson Monoclonal Center, Mountain View, CA, USA
* * ; Nichi-rei, Tokyo, Japan
* * * ; Ortho Pharmaceutical Inc, Laritan, NJ, USA
* * * * ; Coulter Immunology, Hialeah, FL, USA
Lymphoid markers are underlined. Myeloid markers are shaded. Monocyte markers are underlined with broken lines; supplementary markers are not marked.

Table 3. Antigens Used for Lineage Diagnosis of Leukemic cells

Lymphoid antigens
CD2, CD3, CD7, CD10, CD10, CD19, CD20, terminal deoxynucleotydil transferase
Myeloid antigens
CD13, CD33, peroxidase

RESULTS

1. HAL

On the basis of the diagnostic criteria described above, 15 of 29 cases (51%) of acute lymphoblastic leukemia (ALL) and 4 of 43 cases (9%) of acute non-lymphocytic leukemia (ANLL) were diagnosed as HAL. Characteristics of the 17 HAL cases are shown in Table 4.

Table 4. Clincal characteristics, phenotypes and cytochemical features in HAL cases

No.	Age	Sex	Diag	Sample /blast(%)	Type	Lymphoid markers							Myeloid markers			Ph[1]
						CD2	CD3	CD7	CD10	CD19	CD20	TdT	CD13	CD33	POX	
1	2	F	L2	BM/57	I	6	5	6	88	88	86	+	5	94	—	—
2	68	M	L1	PB/49	I	45	18	29	46	50	56	+	9	53	—	—
3	38	M	L2	BM/75	I	6	5	6	85	82	7	+	4	87	—	—
4	69	F	L2	PB/89	I	10	7	17	73	90	11	−	18	44	−	+
5	70	F	L2	PB/86	I	17	13	8	60	61	6	+	17	81	−	+
6	18	F	L2	PB/70	I	10	17	18	77	87	85	+	0	67	−	ND
7	69	F	L1	PB/60	I	17	14	15	65	90	71	+	2	50	−	ND
8	16	M	L2	PB/87	II	53	4	98	0	5	1	−	2	51	−	−
9	30	F	L2	PB/60	II	53	44	44	32	45	10	+	5	74	−	−
10	33	M	L2	BM/86	II	13	6	95	85	9	4	−	0	84	−	−
11	71	M	L2	PB/62	III	22	19	19	76	80	9	+	71	60	−	+
12	1	M	L2	BM/62	III	20	9	11	56	61	5	+	45	41	−	ND
13	14	M	L1	PB/40	III	25	22	25	50	73	3	+	61	70	−	−
14	28	F	L2	BM/51	III	21	21	21	37	57	6	+	96	55	−	−
15	40	F	L2	BM/96	III	1	2	5	98	97	6	+	94	95	−	−
16	34	M	M1	PB/99	III	6	5	5	37	57	2	+	86	73	+	+
17	62	M	M2	BM/40	IV	67	53	59	3	10	11	−	71	60	+	−
18	74	M	M2	BM/40	IV	38	37	32	5	4	17	−	46	54	+	−
19	25	F	M2	PB/60	IV	87	7	41	8	6	10	−	57	90	+	−

ND; not done

2. Classification

We divided HAL into 4 types: Type I, expressing one myeloid antigen (CD33) and 2 or more B-cell antigens, including common ALL antigen (CALLA, CD10); Type II, expressing one myeloid antigen (CD33) and 2 or more T-cell antigens; Type III, expressing two myeloid antigens (CD13 and CD33) and 2 or more B-cell antigens, including CALLA (CD10); and Type IV, expressing at least 2 of 3 myeloid markers (CD13, CD33 and peroxidase) and 2 or more T-cell antigens.

The incidence of Type I was 7 of 15 cases. These Type I cases had CALLA (CD10) and CD19 as B-cell antigens. TdT was positive in 6 of the 7 Type I cases. There were 3 Type II cases, 2 of which had CD2 and CD7 as T-cell antigens, the remaining case being CD7 and CALLA positive. In Type I and II cases, no myeloid antigen other than CD33 was observed. Since peroxidase was also negative, the CD33 antigen meant low specificity as a myeloid marker.

There were 6 Type III cases, all of which had at least two of CD10, CD19 or TdT (lymphoid antigens) and both CD13 and CD33, although peroxidase was negative in all but one case. CD20 was negative in all of them.

There were 3 Type IV cases, 2 of which had 3 T-cell antigens (CD2, CD3 and CD7); the remaining case was CD2 and CD7 positive. All cases had both CD13 and CD33 and were peroxidase positive.

The results, showing typical phenotypes of Types III and IV, are presented in Fig. 1 and 2.

Contour-line presentation of dual-staining with FITC-CD10 and PE-CD33 in Case 15 is shown in Fig. 1, indicating that most leukemic cells had both antigens.

Fluorescence histograms for CD3, CD13 and CD33 in Case 18 are shown in Fig 2. The positive response of this case to each antibody is clear as compared to the negative response in the control IgG test.

DISCUSSION

Phenotypic analysis of leukemic cells with monoclonal antibodies has become essential in the diagnosis of acute leukemia.

A currently available FCM has made it possible to accurately detect weak antigens which could not be detected by the previous type of FCM. Using FACS-

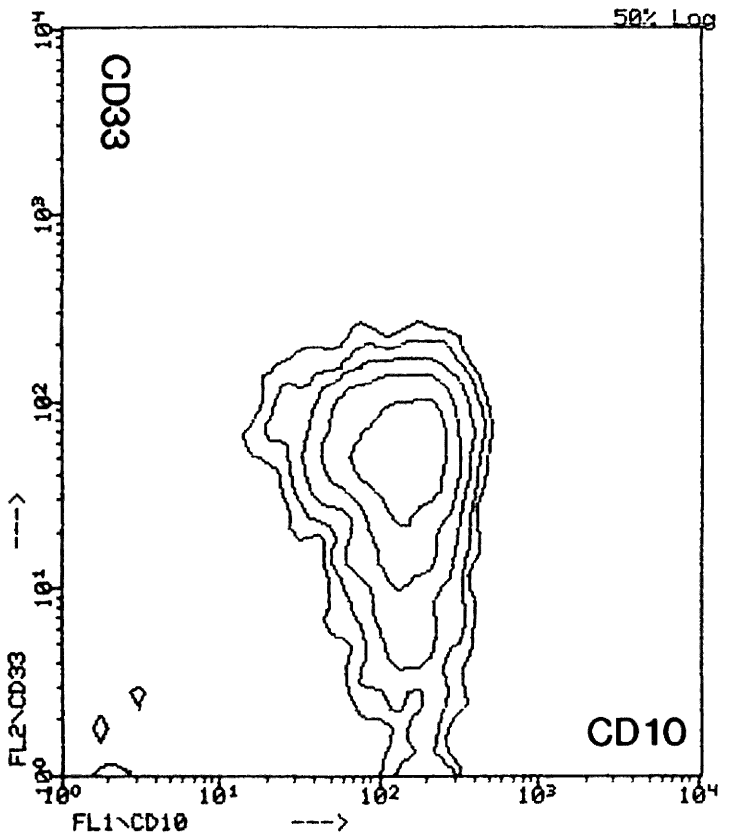

Fig. 1. Double staining of leukemia cells in Case 16 with FITCH10 and PE-CD33. Contour lines show that most leukemic cells have both antigens.

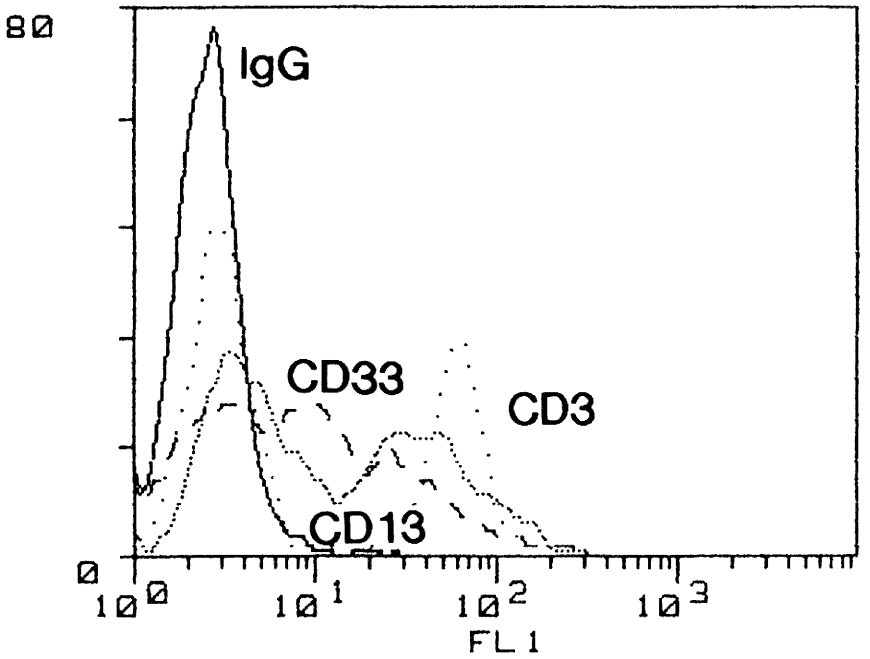

Fig. 2. Fluorescence histograms for CD3, CD13, and CD33 in Case 18. Three antigens are positive in different degrees as compared with the negative control of IgG.

can, investigators have attempted to reclassify the so-called undetermined leukemia or unclassified leukemia.[3,4] The proposal of the term HAL by Gale in 1987 was one result.

The incidence of HAL varies depending on the definition of lymphoid and myeloid antigens. Mirro et al.[8,9] reported that lymphoid antigens were positive in 10 of 53 children (18.9%) with ANLL, and that 8 of them had only CD2 antigen. Cross et al.[10] also described CD2-positive acute myeloid leukemia (AML) with morphologically typical myeloblasts. Kaplan et al. also noted that T-cell antigens were positive in 16 of 55 ANLL cases diagnosed morphologically or cytochemically.[11] The antigens found in these studies were CD2 alone or CD2 and CD4. It did not seem appropriate to regard these reported cases as lymphoid antigen positive ANLLs, since CD2 and CD4 antigens were not specific for lymphoid lineage.[12]

Childs described 14 cases with myeloid antigens out of 40 typical ALL cases (35%).[13] In his cases, only CD33 was used as the myeloid antigen as in our Type I and II cases. Their cytochemical and clinical features are consistent with those of ALL. Since the CD33 antigen was described as being expressed also on lymphoid stem cells,[14] the claim of CD33 as myeloid antigen in the diagnosis of HAL seems to be weak and inappropriate.

Poor responses of CD2-positive ANLL to treatment were describeed by Cross et al.[10] and CD33-positive ALL were therapy-resistant.[13] Particular attention should be paid to these cases, regardless of whether the diagnosis of hybrid leukemia is appropriate or not.

Type III (with two myeloid antigens CD13 and CD33 and at least two B-cell antigens positive) and Type IV (with CD13 and CD33 and multiple T-cell antigens) satisfy the criteria of HAL definition strictly.

The incidence of HAL judged by strict criteria among our cases of acute leukemia was 12.5% (9/72). Since 5 Type III cases except Case 16 showed peroxidase negative and TdT positive, they were cytochemically thought to be ALL. Therefore, Type III seems to represent HAL myeloid-antigen-positive ALL cells. None of the Type III cases were CD20 positive, whereas 4 out of 7 Type I cases were CD20 positive. These results suggest that ALL cells in HAL were in a juvenile stage of B cell lineage without more differentiated B cell antigens. Case 16 was CD19 and TdT positive, but was cytologically diagnosed as ANLL due to its being peroxidase positive.

In Type IV cases, leukemic cells were peroxidase positive and additionally possessed multiple T-cell antigens. Two of them had CD2 antigen as well. Type IV HAL could be regarded as T-cell antigen positive ANLL.

The possibility of making a misdiagnosis of "hybrid leukemia" based on contaminants other than leukemic cells could be ruled out for the following reasons: (1) Gating on the cytogram allowed increases of the ratio of leukemic cells even in bone marrow tests.[1] (2) The use of dual-staining allowed detections of multiple antigens on a single cell. Phenotype analysis by this method was possible when the percentage of blasts in the total leukocytes was over 30%.[1,6]

In our study, cases with 40% or more blasts in total leukocytes were analyzed. In these HAL cases, the analysis of surface markers alone did not allow us to make a definite identification of lineage. However, it seemed more acceptable to explain these cases on the concept of lineage promiscuity (i.e., the coexistence of juvenile markers) proposed by Greaves et al.[12] rather than on the conventional concept of lineage infidelity.[4] The new concept was indirectly supported by: (1) Blasts in cases of HAL appeared to be at quite a juvenile stage; and (2) Philadelphia (Ph^1)-chromosome positive acute leukemia was sometimes seen in cases of HAL. The presence of these cells does not contradict the view of Ogawa et al. that cells in the early stage of differentiation have the potential to differentiate into any lineage.[15]

Evaluation of the clinical significance of HAL requires the accumulation of cases and follow-up studies. Sobol et al.[16] reported that the complete remission rate in myeloid antigen positive ALL was significantly lower in common ALL. A similar result was reported in the study of 40 cases of myeloid antigen positive ALL by Childs.[13] More recently, Mirro et al. found that the presence/absence of myeloid antigens did not correlate with the prognosis of children with ALL.[17] However, we have to be careful in interpreting Mirro's results, since various myeloid antigens, including those without clear specificity, were treated and discussed together in their study.

Based on these findings, we should consider HAL in selecting a therapy for acute leukemia, and that additional cases of HAL should be studied using methods of molecular biology as well.

CONCLUSION

Dual-staining of leukemic cells revealed that 9 of 72 cases (12.5%) with acute leukemia satisfied the strict

criteria of HAL, these definitely possessing both myeloid and lymphoid markers. The response of HAL to treatment could be different from that of conventional acute leukemia. Further study is needed to clarify the clinical significance of HAL.

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