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Phenotype Study of Fresh and Cultured Hairy Cells With the Use of Immunologic Markers and Electron Microscopy

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The phenotype of fresh and cultured leukemic cells from patients with hairy cell leukemia was studied using a panel of monoclonal antibodies in addition to the detection of peroxidase activity under electron microscopy. In fresh samples, the leukemic cells from 11 patients displayed predominantly a B phenotype, as judged by their reactivity with the B1 monoclonal antibody and surface immunoglobulin expression. Ultrastructural peroxidase activity, characteristic of hairy cells, was observed in all cases studied. When hairy cells were cultured in the presence of phytohemagglutinin and irradiated T cells, their phenotype converted from surface Ig^+, $B1^+$, $OKT3^-$, $OKT11^-$ to surface Ig^-, $B1^+$, $OKT3^-$, $OKT11^+$. In contrast, the peroxidase activity remained unchanged. Some hairy cells were also $OKM1^+$, but no conclusion could be made about the MO_2 antigen, a more specific marker of monocytes. The variability of the phenotype in vivo and in vitro indicates that reliable markers are required for identifying hairy cells. When studied together, the staining by B1 monoclonal antibody and the ultrastructural detection of peroxidase, enable the identification of hairy cells with certainty.

THE ORIGIN of the leukemic cells in hairy cell leukemia (HCL) remains unknown because of the absence of an identified normal counterpart in the hematopoietic system. Hairy cells (HC) express both lymphocytic and monocytic features.[1,2] They are clearly related to the B lymphocyte lineage by their ability to rearrange the DNA of genes coding for immunoglobulin heavy and light chains.[3] Also, HC demonstrate a peroxidase activity of monocytic type that is detectable under electron microscopy.[4] Furthermore, the capacity of HC to form spontaneous rosettes with sheep red blood cells, like T lymphocytes, is not uncommon.[5,6] In vivo, the fluctuations of HC surface B and T characteristics have been described.[7,8] In vitro, HC cultured in the presence of phytohemagglutinin (PHA) and allogeneic T lymphocytes acquire receptors for sheep erythrocytes (E receptor) and lose surface immunoglobulin.[9,10] These observations underline the variability of the HC phenotype, which has led us to search for reliable markers to identify HC.

Using a set of monoclonal antibodies in conjunction with ultrastructural cytochemistry, we show that the peroxidase activity specifically recognizes HC among leukemic cells with B lymphocyte characteristics. The B cell characteristic is demonstrated by staining with the B1 monoclonal antibody,[11] in vivo and in vitro, whether surface Ig are detectable or not. Finally, the possible acquisition of the E receptor by HC seems to be a more general feature of B cells. It has been found for normal B lymphocytes[12] and for B cells in chronic lymphocytic leukemia (CLL)[13,14] and chronic prolymphocytic leukemia (PLL), as shown in the case included in the present study.

MATERIALS AND METHODS

Patients

Cells from 11 patients with hairy cell leukemia (HCL) were studied. The diagnosis had been suggested by a compatible clinical picture and the presence in peripheral blood of typical hairy cells (HC). The diagnosis was confirmed, in all instances, by the characteristic histopathologic findings in bone marrow, and in the liver and spleen of splenectomized patients. None of the patients had received steroids or cytotoxic therapy prior to the study. The various blood and/or spleen specimens investigated for surface phenotype and cytochemistry are listed in Table 1. Included in this study is a splenectomized patient with a B cell chronic PLL, with $3 \times 10^5/\mu L$ prolymphocytes in peripheral blood.

Cell Suspensions

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. Spleen specimens were teased through a stainless steel mesh sieve to obtain a single-cell suspension and subsequently were centrifuged on Ficoll-Hypaque. HC enrichment from peripheral blood and/or spleen specimens was obtained by T cell depletion after rosetting with sheep erythrocytes (E cell depletion). Allogeneic T cells were obtained from healthy donors and were irradiated (3,000 rad).

Cell Cultures

The various cell fractions, as described above, were cultured at a cell concentration of 10^6/mL in RPMI 1640 medium (Eurobio, Paris) supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), with or without 3 μg/mL PHA (PHA-P, Difco, Detroit, MI), in the presence or in the absence of 20% normal allogeneic irradiated T cells.[10] Cultures were carried out in plastic flasks (Corning, Corning, NY), incubated for four to seven days at 37 °C in 6.5% CO_2 and 100% humidity.

Cell Phenotype Determination

Surface Ig was identified by direct immunofluorescence using FITC-labeled rabbit anti-heavy and anti-light chains (Dakopatts, Glosdrup). Cell staining by monoclonal antibodies was performed by an indirect technique using FITC-labeled goat anti-mouse $F(ab')_2$

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Table 1. Phenotype of Fresh Mononuclear Cells From Peripheral Blood and/or Spleen in 11 HCL Cases

Patient	Specimen	Percent of Labeled Cells						
		sIg	B1	OKT3	OKT11	OKM1	MO_2	Peroxidase
1	PB	63 μλ	66	28	31	1	ND	90
	PB*†	69 μλ	75	24	28	73	2	ND
	PB*	88 μλ	93	6	5	90	5	ND
2	PB	27 μλ	27	52	51	49	29	ND
3	PB	8 μλ	10	ND	70	1	1	ND
	PB*	70 μλ	75	8	13	1	1	ND
4	PB	65 μλ	66	ND	28	1	1	ND
	PB‡	40 μλ	45	51	50	42	38	ND
	PB*‡	90 μλ	94	2	2	84	ND	72
	SP	82 μλ	73	20	10	6	10	74
5	SP	61 γκ	59	40	30	8	9	85
6	SP*	90 γκ	88	3	4	7	7	82
7	SP	70 γκ	58	33	26	15	9	80
8	SP	62 μλ	78	22	20	3	3	80
9	SP	35 γκ	24	64	76	36	20	ND
10	SP	ND	62	ND	17	10	12	ND
11	SP	64 μλ	85	10	19	83	1	80

SP, spleen; PB, peripheral blood; ND, not done.

*E-depleted cells.

†Second determination six months apart.

‡After splenectomy.

fragments (Cappel, Cochranville, Pa). Reactivity pattern of the various antibodies is as follows: OKT3 stains mature T cells,[15] and OKT11 reacts with the E receptor;[16] B1 specifically identifies B lymphocytes;[11] OKM1 stains monocytes, granulocytes, and a subpopulation of T cells;[17,28] MO_2 is specific for monocytes;[18] J15, antiglycophorin, and 80H5 react with determinants expressed by the megakaryocytic,[19] erythrocytic,[20] and granulocytic[21] lineages, respectively.

Controls for nonspecific binding of monoclonal antibodies included cell staining by an irrelevant first layer (anti-poly [Glu^{60}, Ala^{30}, Tyr^{10}] antibody)[22] and by the FITC-labeled $F(ab')_2$ second layer alone. The slides were examined under a Zeiss episcope equipped with phase-contrast. At least 250 cells were counted per slide. Phenotypic data on day 0 are those of fresh HC that were further cocultured with irradiated T cells; these data did not include irradiated T cell phenotype, as these cells die within 72 hours.

Peroxidase Activity

Unfixed cells were incubated in a diaminobenzidine (DAB)-H_2O_2 containing medium, postfixed, and processed to embedding as previously described.[4] The percentage of positive cells was determined under electron microscopy by counting at least 200 cells.

RESULTS

Phenotype of Fresh HC

Peroxidase activity was detected in HC in the eight specimens studied by ultrastructural cytochemistry. The enzymatic activity was localized in the perinuclear space and endoplasmic reticulum; Golgi apparatus and granules were negative.[4] Peroxidase-positive HC were found in peripheral blood, in spleen, and in peripheral blood after splenectomy (Table 1). Usually, no monocytes, whose peroxidase content is restricted to granules, were observed under electron microscopy. When present, they did not exceed 2% of the preparations studied.

In all specimens, the cells that looked hairy by phase-contrast microscopy stained positively with the B1 monoclonal antibody and expressed surface Ig with a monotypic light chain. Although no double-staining experiments were performed, the B1 and surface Ig^+ cells were the same for the following: (a) the number of B1 and surface Ig reactive cells was similar in all cases, with a significant overlap in percentages in seven cases; (b) the percentages of $B1^+$ and peroxidase-positive cells also overlapped; (c) after E cell depletion, the number of surface Ig^+, $B1^+$ and, peroxidase-positive cells still coincided. Thus, hairy cells were B1-positive in the samples studied.

The proportions of $OKT3^+$ and $OKT11^+$ cells were highly variable from one patient to another, but were similar in a given specimen. These cells did not look hairy by phase-contrast microscopy, suggesting that these antibodies identified normal T cells (Table 1). The OKM1 antibody stained 49% of peripheral blood cells in patient 2; and more than 70% of peripheral blood cells in specimens from patients 1 and 4.

Thirty-six percent and 83% of spleen cells were $OKM1^+$ in patients 9 and 11, respectively. In patient 1, spontaneous variation in OKM1 positivity was observed, and in patient 4, $OKM1^+$ cells were detected only after splenectomy. The sum of $B1^+$ and $OKM1^+$ cells exceeded 100% in three cases and that of peroxidase-positive and $OKM1^+$ cells exceeded 100% in two cases (Table 1). Consequently, a few HC appeared to

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Table 2. Phenotypic Change in Unfractionated HCL Suspensions in the Presence of PHA and Irradiated T Cells

Patient	Specimen	Day	Percent of Labeled Cells						
			sIg	B1	OKT3	OKT11	OKM1	MO_2	Peroxidase
1	PB	0	63 μλ	66	28	31	1	ND	90
		7	23 μλ	68	33	75	65	ND	66
4	SP	0	82 μλ	73	20	10	6	10	74
		7	12 μλ	74	32	52	56	5	65
5	SP	0	61 γκ	59	40	30	8	9	85
		6	9γκ	41	61	93	ND	ND	57
7	SP	0	70 μκ	58	33	26	15	9	80
		5	2γκ	45	53	62	13	0	54
8	SP	0	62 μλ	78	22	20	3	3	80
		6	10 μλ	55	33	62	20	ND	65

Abbreviations as in Table 1.

be $OKM1^+$. A significant percentage of MO_2^+ cells was also detected in peripheral blood cells from patient 2, from patient 4 after splenectomy, and in spleen cells from patient 9. However, these specimens were characterized by a low number of $B1^+$ cells, with no overlap in percentages. The high number of $B1^+$ and $OKM1^+$ cells contrasted with the low number of MO_2^+ cells in patients 1 and 11 (Table 1). Whereas some HC were OKM1-positive, they were MO_2 negative. In no specimens were 80H5, J15, and antiglycophorin antibodies found to stain HC. Finally, in vivo, the majority of HC displayed a peroxidase-positive, surface Ig^+, $B1^+$, $OKT3^-$, $OKT11^-$ phenotype, some HC being, in addition, $OKM1^+$.

Phenotype of Cultured HC

After culture, with or without PHA and in the presence or in the absence of allogeneic irradiated T cells, a peroxidase activity was detected in the cultured cells of the seven specimens studied by ultrastructural cytochemistry (Tables 2 and 3). The distribution of peroxidase staining was restricted to the endoplasmic reticulum and perinuclear space, which allowed the identification of HC among cultured cells. Ultrastructural examination of cultured HC provided two additional data: (a) proliferation of HC was unlikely, as no mitoses were detected, and (b) there was no evidence of HC maturation into plasma cells, ie, no enrichment in polyribosomes and in endoplasmic reticulum channels and no enlargement of the Golgi apparatus.

In contrast to the persistent expression of the peroxidase activity, the reactivity of HC with immunologic markers showed marked fluctuations. However, no phenotypic change occurred when unseparated or E cell-depleted HC suspensions were cultured, either alone, with or without PHA, or in the presence of allogeneic irradiated T cells without PHA (Table 4). In contrast, a phenotypic alteration was observed in unseparated (Table 2) and in E cell-depleted HC suspensions (Table 3) when cultured in presence of both PHA and allogeneic irradiated T cells. Whereas virtually all cells that looked hairy by phase-contrast microscopy became surface Ig^-, they remained strongly $B1^+$. In addition, a large number of unfractionated mononuclear cells became $OKT11^+$, whereas the proportion of $OKT3^+$ cells remained almost unchanged, except in cases 5 and 7 (Table 2). In the latter, the increase in OKT11 positivity could not be clearly related to normal T cell proliferation or to a change in HC phenotype. Therefore, HC suspensions were depleted of autologous T cells by the E cell depletion method described above. Such HC-enriched fractions that contained few $OKT3^+$ cells (Table 3) were cultured with irradiated allogeneic T cells. A

Table 3. Phenotypic Change in E-Depleted Fractions of HCL Suspensions Cultured in the Presence of PHA and Irradiated T Cells

Patient	Specimen	Day	Percent of Labeled Cells						
			sIg	B1	OKT3	OKT11	OKM1	MO_2	Peroxidase
1	PB	0	88μλ	93	6	5	90	5	ND
		4	2 μλ	93	10	63	85	2	ND
3	PB	0	70 μλ	75	8	13	1	1	ND
		6	20 μλ	80	20	50	1	1	ND
4	PB*	0	90 μλ	94	2	2	84	ND	72
		7	7 μλ	86	8	70	56	ND	78
6	SP	0	90 γκ	88	3	4	7	7	82
		6	15 γκ	82	24	53	5	0	47

Abbreviations as in Table 1.

*After splenectomy.

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Table 4. Surface Phenotypic Findings in E-Depleted Blood Mononuclear Cells (E^- HC) From HCL Case 1, Cultured Under Various Conditions

Specimen	Day	Percent of Labeled Cells					
		sIg	B1	OKT3	OKT11	OKM1	MO_2
E^- HC	0	88 μλ	93	6	5	90	5
E^- HC	4	95 μλ	95	<2	<2	90	<2
E^- HC + PHA	4	90 μλ	90	8	10	88	<2
E^- HC + irradiated allogeneic T cells	4	95 μλ	98	<2	<2	98	<2
E^- HC + PHA + irradiated allogeneic T cells	4	2 μλ	93	10	63	85	<2

striking rise in $OKT11^+$ cells was still observed under these conditions. The high number of $OKT11^+$ cells did not relate to the persistence of irradiated T cells after culture, as the latter cultured alone died and the number of $OKT3^+$ cells remained lower than 25% (Table 3). Finally, the comparison of the different percentages of peroxidase-positive, $B1^+$, and $OKT11^+$ cells indicated that HC acquired OKT11 reactivity in the presence of PHA and irradiated T cells.

$OKM1^+$ cells emerged in the culture of unseparated peripheral blood cells from patient 1 and spleen cells from patient 4 (Table 2). In these cases, the percentage of $OKM1^+$ cells overlapped that of peroxidase-positive and $B1^+$ cells. In contrast, less than 5% MO_2^+ cells were observed in the various cultured specimens (Tables 2 and 3). Thus, when HC were cultured with both PHA and irradiated T cells, they expressed a peroxidase-positive, surface Ig^-, $B1^+$, $OKT3^-$, $OKT11^+$ phenotype, some HC being, in addition, $OKM1^+$.

Phenotype of Cultured Prolymphocytes

An alteration of the surface phenotype has been reported in culture of normal[12] and CLL B cells.[13,14] When cultured in the presence of PHA and irradiated T cells, such B lymphocytes coexpress the E receptor and surface Ig. Therefore, to question whether the disappearance of surface Ig in culture was restricted to HC, we also studied a specimen of leukemic B cells from a patient with PLL.

In vivo, PLL cells contained no peroxidase activity detectable under electron microscopy; their surface phenotype was surface Ig^+, $B1^+$ (Table 5). No phenotypic conversion occurred in culture when PHA and/or irradiated T cells were omitted. However, when PLL cells were cultured in the presence of both PHA and irradiated T cells, there was a sharp increase in $OKT11^+$ cells, which contrasted with the low number of $OKT3^+$ cells (Table 5). The PLL cells remained $B1^+$ and, in contrast to HC, surface Ig^+. The overlap among the percentages of surface Ig^+, $B1^+$ and $OKT11^+$ cells showed that cultured PLL B cells coexpressed these three markers.

DISCUSSION

In order to better define reliable markers for the identification of HC under various microenvironmental conditions, we studied the phenotype of fresh and cultured HC using monoclonal antibodies in addition to ultrastructural cytochemistry.

In vivo, HC expressed a peroxidase activity revealed under electron microscopy and a surface Ig^+, $B1^+$, $OKT3^-$, $OKT11^-$ membrane immunologic phenotype. The peroxidase activity was located exclusively in the endoplasmic reticulum and perinuclear space; no positive granules were associated with this pattern. In contrast, monocytes have myeloperoxidase-positive granules and unstained reticulum strands, which allows a clear distinction between HC and rate contaminating monocytes.[4] However, the cytochemical pattern of HC peroxidase has been show to be similar to that of the peroxidase activity, which appears in adherent monocytes, in addition to the myeloperoxidase of granules. The staining pattern of HC peroxidase is also similar to that found in the megakaryocytic lineage.[19] In contrast, this enzymatic activity, found in some normal myeloid cells, has not been detected in leukemic nor normal T and B lymphocytes.[4] HC expressed the surface B1 determinant, a differentiation antigen characteristic of the B cell lineage. The B1 monoclonal antibody identifies normal and leukemic B cells at various stages of maturation.[23] The presence of the B1 antigen at the surface of HC is in concordance

Table 5. Phenotypic Findings in Leukemic Prolymphocytes Cultured in the Presence of PHA and Irradiated T Cells

Specimen	Day	Percent of Labeled Cells						
		sIg	B1	OKT3	OKT11	OKM1	MO_2	Peroxidase
Pheripheral blood	0	92 μκ	98	2	2	0	0	0
mononuclear cells	5	90 μκ	95	10	60	0	0	0

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with the presence, in vivo, of a monotypic surface Ig. Although we did not perform double-labeling experiments, the data indicate that HC do not possess the T3 determinant, a structure associated with the T cell antigen receptor;[24] neither do HC seem to express in vivo the E receptor detected in this study by the OKT11 monoclonal antibody. Occasionally, the OKM1 antibody was found to stain HC, in agreement with other data.[25,26]

This antibody has been shown to react with the C3 bireceptor on mononuclear phagocytes,[27] but its reactivity is not restricted to the monocyte lineage.[28] MO_2 reactivity, a more specific monocyte marker, was found in low numbers of cells in most specimens, which does not allow any formal conclusion as to whether a few HC express monocytic determinants on their membrane or not. Consequently, the HC phenotype in vivo consisted of the surface characteristics of the B lymphocyte lineage in addition to a particular peroxidase activity normally present in some myeloid cells at defined stages of their maturation.

In vitro, the HC continued to synthesize the peroxidase activity under all culture conditions studied. The surface phenotype also remained unchanged under most conditions, except when HC were cocultured with both allogeneic irradiated T lymphocytes and PHA. Thus, whereas the peroxidase activity persisted, their immunologic phenotype switched from surface Ig^+, $B1^+$, $OKT3^-$, $OKT11^-$ to surface Ig^-, $B1^+$, $OKT3^-$, $OKT11^+$. This phenotypic alteration did not result from the emergence of a proliferative HC subset, as no mitoses were observed under electron microscopy. The disappearance of surface Ig could not be explained by a masking effect of the mitogen, as shown by the surface Ig positivity in control cultures of HC with PHA alone (Table 4). Another explanation for the disappearance of surface Ig in culture would be that HC undergo plasma cell maturation. We found no morphological evidence of such a maturation under electron microscopy, that is, no development of the organelles involved in Ig synthesis. Furthermore, Guglielmi et al[9] have shown the reversibility of this phenomenon, with reexpression of surface Ig within a few hours, when cultured HC are reincubated in fresh medium with no PHA and no T cells. Also the B1 antibody is still reactive on cultured HC, whereas it does not stain plasma cells from myeloma patients.[23] Therefore, HC did not demonstrate a plasma cell phenotype in culture. The disappearance of surface Ig seems to be characteristic of cultured HC, as PLL cells (shown here), CLL cells,[13,14] and normal B cells[12] continue to display surface Ig in addition to expressing the E receptor under similar conditions. It is unlikely that surface Ig of HC would be masked by a T cell product in the presence of PHA: this possibility should be independent of the B cell type of cultured cells. Another possibility would be a modulation of surface Ig triggered by T cells in the presence of PHA. This hypothesis is strongly suggested by the reversibility of the phenomenon and is compatible with a restriction to HC, as the modulation capacity has been shown to depend on membrane maturation in the B cell lineage.[29]

HC acquired the E receptor when cultured with both PHA and allogeneic T cells. In contrast to the disappearance of surface Ig, the acquisition of E receptor is not restricted to HC: it has been demonstrated for normal B lymphocytes,[12] for CLL B cells,[13,14] and for PLL B cells in our study. Whether the significance of this finding remains unknown, neither the HC nor the other B cell types acquire other T lymphocyte differentiation antigens, such as OKT3. The ability for HC to form E rosettes or to express the OKT11 determinant may account for previous reports of T cell cases of HLC[5,6] in the absence of more specific markers. Consequently, the membrane phenotype of HC in culture can be modified by the cellular environment, but remains that of a B cell with no T cell-specific markers. Although OKM1 positively could be induced on some cultured HC, the staining by MO_2 remained negative. Thus, no consistent monocyte-specific surface phenotype was found on cultured HC.

In conclusion, the HC phenotype appears to be variable in vitro and occasionally in vivo: possible disappearance of surface Ig and expression of the E receptor could suggest an erroneous T cell nature for the leukemic cells. Our study has shown that the use of the B1 monoclonal antibody is necessary for demonstrating their B lymphocytic characteristic in the absence of detectable surface Ig. In addition, the detection of peroxidase activity under electron microscopy is a reliable procedure for identifying hairy cells among leukemic B cells.

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