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Cell-Specific Polymorphism of Acid Phosphatase in Human Blood Cells: Their Functional and Leukemic Variants[1] (41186)

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Abstract. The observation that the cytochemical patterns of certain enzymes represent a stable marker for the identification of blood cells and resist functional stimuli as well as malignant transformation prompted the study of cell-specific polymorphism of acid phosphatase (EC 3.1.3.2) in human blood cells. Normal and leukemic human T and B lymphocytes as well as normal and leukemic blood monocytes and human alveolar macrophages as a functional derivative of blood monocytes were separated and subjected to purity control. Lysosomes, collected after cell cavitation, were solubilized and the particle-free supernatant was subjected to a direct assay of acid phosphatase using *p*-nitrophenylphosphate. Aliquot samples were analyzed by thin-layer isoelectric focusing and the banding of the acid phosphatase was recorded on gels using naphthol AS–BI phosphate after having demonstrated that on a quantitative term a positive correlation exists between the results with both substrates. The banding patterns proved to be highly cell specific as far as blood cells were concerned. The cell specificity of the acid phosphatase polymorphism defied functional as well as neoplastic transformation allowing for a clear recognition and classification of the individual cell lines. The cell-specific polymorphism of lysosomal enzymes seems to bear the potentiality of a further biochemical cell marker as exemplified by the results obtained with acid phosphatase.

Acid phosphatase (EC 3.1.3.2) assessment, for a long time confined to the clinical detection of prostatic carcinoma (1) and disorders of lysosomal system (2), has recently attracted considerable attention in context with neoplasias of the hematopoietic system (3, 4). Whereas monocyte–macrophage cell lines show a high cellular activity by cytochemical tests as well as by direct measurement (5), the activities found in other blood cell types are of lower order (6, 7). In spite of the extremely low enzyme content, different lymphocyte subpopulations show a characteristic cytotopographical distribution of acid phosphatase (AcP) activity (8). Human B lymphocytes, being mostly enzyme negative, might be presented with a weak diffuse cytochemical enzyme pattern. T lymphocytes are regularly enzyme positive. T helper lymphocytes are specifically characterized by an enzyme activity confined to one or two coarse granular structures. T suppressor lymphocytes on the other hand have a cell-specific granular enzyme pattern, evenly distributed throughout the cytoplasm.

Because the cytochemical pattern of AcP remains fairly constant within individual blood cell types resisting functional stimuli or even neoplastic transformation, the question was raised whether the cytochemical patterns might conform to a cell-specific enzyme polymorphism, detectable not only in normal blood cell types but also in their functional derivatives and neoplastic variants. In the present study the polymorphism of the lysosomal AcP of purified normal human blood cells as detected by thin-layer isoelectric focusing (IEF) will be compared with the patterns of alveolar macrophages (AM) as a functional form of blood monocytes, leukemic monocytes, and leukemic T and B lymphocytes.

Materials and Methods. (*1*) *Cell separation.* Heparinized venous blood from healthy young donors was used. Following centrifugation on a Ficoll–Urografin gradient (Pharmacia, Uppsala, Sweden; Schering, Berlin, West Germany) adjusted to $D = 1.007$ g/ml (9) granulocytes were

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obtained from the bottom fraction after osmotic lysis of the erythrocytes. Monocytes were separated from the mononuclear cells of the interphase by glass adherence (10). Nonadherent cells were passed through an anti-human immunoglobulin column (11), packed with polymethylmetaacrylic beads (Degussa, Hanau, West Germany) coupled with human IgG, anti-human rabbit IgG, and human C3; only T lymphocytes passed the column, whereas B lymphocytes as well as nonadherent monocytes were retained. Platelets were gathered from platelet-rich plasma by centrifugation (400*g*, 20 min, 20°) and erythrocytes were isolated by ultracentrifugation as described by Fenton and Richardson (12).

B lymphocytes were isolated from minced and sieved fresh human tonsils. Contaminating T lymphocytes were excluded after rosetting of the tonsillar cell suspension with 2-aminoethylisothiouronium bromide (Sigma, Munich, West Germany)-treated sheep erythrocytes (13) followed by repeated centrifugation on Ficoll–Urografin gradient (14), and discarded. If necessary, monocytes, macrophages, and other tissue-resident cells were removed by glass adherence.

Human alveolar macrophages were collected from surgically ectomized lungs due to bullous emphysema or bronchial neoplasm. For this purpose, pulmonary lobes, not affected by carcinoma or inflammatory foci, were repeatedly rinsed with saline and mononuclear cells were separated as indicated above.

In addition, three cases of monocytic leukemia, three patients with chronic T lymphocytic leukemia, and two patients with chronic B lymphocytic leukemia were investigated. Some hematological data of these patients are surveyed in Table I.

In further analyses only purified cell suspensions with a viability rate exceeding 95%, tested by trypan blue exclusion, were used.

The purity of isolated cell fractions was established by differential counting of cytocentrifuge preparations (Shandon, London, England). Neutrophils were selectively visualized by the naphthol AS–D chloroacetate reaction (15). T lymphocytes, monocytes, and macrophages were identified using the α-naphthylacetate esterase method according to Mueller *et al.* (16). In addition the lymphocyte subpopulations as well as chronic lymphocytic leukemias were checked by immunological methods. The rosetting test with neuraminidase (Behring, Marburg, West Germany)-treated sheep erythrocytes (17) was used for the characterization of T-cell lineage. B-Lymphocytic nature was established by an immunofluorescence method using rhodamine isothiocyanate (Nordic, Tilburg, The Netherlands)-conjugated polyvalent rabbit anti-human Ig sera (18).

(*2*) *Assay of AcP activity.* AcP activity was measured using commercial kits (Boehringer, Mannheim, West Germany) with *p*-nitrophenylphosphate as substrate (19). The enzyme assay was performed on the lysosomal fraction, which was obtained

TABLE I. HEMATOLOGICAL DATA OF THE LEUKEMIC CASES INVESTIGATED

Leukemia	Case No.	Age	Sex	WBC ($\times 10^3$ cells/μl)	Percentage leukemic cell type	SE_N (%)	SIg (%)
Monocytic	1	64	m	115	70	n.d.	n.d.
	2	50	m	97	96	n.d.	n.d.
	3	76	f	34	50	n.d.	n.d.
Chronic T lymphocytic	1	61	f	384	96	78	4
	2	63	f	212	94	89	1
	3	71	m	500	96	92	2
Chronic B lymphocytic	1	62	m	30	91	4	93
	2	69	m	142	92	1	60

Note. WBC, white blood cell count; SE_N, rosetting with neuraminidase-treated sheep erythrocytes; SIg, surface immunoglobulins.

after subjecting the cell suspensions to a cell cavitation bomb (Parr Instruments, Moline, Ill.) and differential centrifugation (20).

Because over 97% of AcP activity is localized in the lysosomal fraction, some of the separated cell suspensions were directly treated with 1% (v/v) Triton X-100 (Serva, Heidelberg, West Germany) for 30 min at room temperature. The activities, measured in the supernatants after ultracentrifugation (100,000*g*, 60 min, 4°) showed no significant difference from those values obtained in the lysosomal fractions. The enzyme activity was expressed in mU/1 × 10^7 cells.

(*3*) *Thin-layer isoelectric focusing.* The IEF was carried out on 4.3% polyacrylamide (Canalco, Rockville, Md.) thin-layer slabs (25 × 15 × 0.3 cm) containing 13.3% (wt/v) sucrose, 1% (v/v) Triton X-100, 0.057% (wt/v) riboflavin, 5% (v/v) ampholyte (LKB, Bromma, Sweden, pH 3.5–10.0), 0.36% (v/v) ampholyte (pH 4.0–6.0), and 0.36% (v/v) ampholine (pH 5.0–7.0). As anolyte and catholyte 1 M phosphoric acid and 1 M sodium hydroxide were used, respectively. The initial voltage of 200 V was raised to a terminal voltage of 1200 V during 4 hr at 4° (21). A minimum of 8 mU of enzyme activity was applied for each run. This amount of enzyme activity prevents overlooking weak bands and renders optimal resolution of the bands as ascertained in prior experiments.

The enzyme variants were visualized by the histochemical method (22): naphthol AS–BI phosphate (Sigma) and hexazotized pararosanilin were used as substrate and coupler, respectively. The reaction pH was 5.0. The reaction time, beyond which no additional bands could be detected, was 2 hr at room temperature. The gels were photographed as well as densitometrically evaluated at 485 nm (Flying Spot TLP 100, Vitatron, Cologne, West Germany).

The net enzyme activity measured by the substrate *p*-nitrophenylphosphate does not necessarily reflect the levels observed with naphthol AS–BI phosphate used for visualization of enzyme bands on gel slabs. To measure the degree of correspondence between the values obtained with both substrates, the following trial was performed: Different samples of AM and human splenic lymphocytes were subjected to a direct enzyme measurement as well as IEF. The latter was evaluated densitometrically. The values were correlated and expressed in terms of correlation coefficient (Fig. 1).

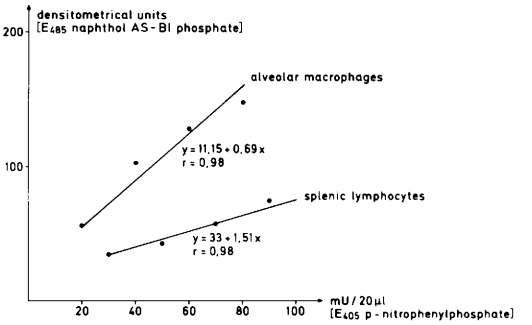

FIG. 1. Correlation curve between AcP activity, measured with *p*-nitrophenylphosphate as substrate, and the tinctorial density of AcP, determined using naphthol AS–BI phosphate.

Results. (*1*) *Cell count and purity.* The total cell count obtained from each separation amounted to 1 × 10^9 for granulocytes, 1 × 10^8 for monocytes, and 2 × 10^8 for T lymphocytes. From five tonsils about 1 × 10^9 B lymphocytes could be separated. The average number of AM collected was 5 × 10^8. Table II shows the differential cell count for the normal cell populations and AM. Granulocytes, T lymphocytes, B lym-

TABLE II. PURITY CONTROL OF SEPARATED HUMAN BLOOD CELLS AND ALVEOLAR MACROPHAGES

Cell type	Percentage
Monocytes	75.6 ± 9.9[a]
Granulocytes	99.0 ± 0.5[a]
T lymphocytes	100.0[a]
	84.5 ± 8.5[b]
	99.0 ± 0.8[c]
B lymphocytes	99.0 ± 0.3[a]
	98.0 ± 1.0[b]
	96.0 ± 1.0[c]
Alveolar macrophages	95.0 ± 3.0[a]

Note. Mean percentage ± SD of four to six different separations. [a–c] Indicate methods of cell identification used in addition to conventional cytomorphology.

[a] Identified by cytochemical methods.

[b] Identified by rosetting test with neuraminidase-treated sheep erythrocytes.

[c] Identified by surface immunoglobulins.

phocytes, erythrocytes, and platelets showed a purity of more than 98%. Because the enzyme activity of monocytes was five to seven times higher than those of B and T lymphocytes, the relative high contamination of monocyte suspensions by lymphocytes was negligible. Leukemic samples consisted almost completely of the proliferating cell type (Table I).

(2) *Enzyme activity.* The activity of AcP per 10^7 cells in the analyzed cell populations is demonstrated in Table III. Monocytes showed the highest values with a mean of $14.0/10^7$ cells as compared with the other normal blood cell types. Significantly lower values were found in granulocytes (5.8 ± 3.4 mU) as well as T and B lymphocytes with 2.8 ± 1.2 and 1.8 ± 1.3 mU, respectively. The lowest content of AcP was detected in platelets. Since the isoelectric focusing pattern of erythrocytes could not be established due to their failure to hydrolyze naphthol AS–BI phosphate, their enzyme activity was not further considered. AM produced values seven times higher than their normal counterpart of blood monocytes. The values for leukemic monocytes and T and B lymphocytes amounted to 17.05, 2.65, and 4.93, respectively.

(3) *IEF pattern of normal cells.* For each category of cells the IEF patterns were found to be constant. Minor differences with respect to the relative intensity and the isoelectric points (p*I*) of the bands were mostly due to technical irregularities, such as hydration of the gels. In Fig. 2 replicate isoenzyme patterns of granulocytes from four experiments are shown demonstrating the reproducibility of the method used.

The typical IEF patterns of AcP for the different normal cell types are surveyed in Figs. 3 and 4; the p*I* values for the different bands represent mostly mean values of four to six different runs.

Monocytes showed three bands between pH 6.3 and 6.0 and eight bands between pH 4.95 and 4.0 (Fig. 3a). Granulocytes revealed 12 enzyme loci, distributed evenly between pH 6.3 and 4.6 (Fig. 3b). Platelets displayed, in addition to the three cathodic bands of monocytes and granulocytes between pH 6.3 and 6.0, one weak band at pH 5.95 (Fig. 3c). T and B lymphocytes were characterized by a strong solitary band at pH 6.3 in contrast to the complex of 3 loci seen in this region in cases of myelogenous cell lines. Whereas T lymphocytes sometimes demonstrated a second band of low intensity at pH 6.15 (Fig. 4a), B lymphocytes showed, in addition to the cathodic solitary band, 11 loci between pH 5.3 and 3.8 (Fig. 4b). As stated above, the AcP of erythrocytes did not hydrolyze the employed substrate.

(4) *IEF patterns of AM and leukemic cells.* AM presented a total of 19 bands. The 11 loci seen in normal blood monocytes were fully present in all runs performed from AM. In addition eight new isoenzymes were localized at pH 5.83, 5.72, 5.5, 5.22, 5.11, 5.0, 4.84, and 4.29 (Fig. 5a).

Leukemic monocytes demonstrated a

TABLE III. AcP Activity ($\bar{X}$ ± SD) in the Separated Cell Populations

Cell type	AcP activity (mU/l × 10^7 cells)	
Monocytes, normal	14.08 ± 6.6	(5)
Alveolar macrophages	106.20 ± 25.1	(3)
Monocytes, leukemic	17.05 ± 7.5	(3)
Granulocytes	5.85 ± 3.4	(6)
T lymphocytes, normal	2.85 ± 1.2	(4)
T lymphocytes, leukemic	2.65 ± 1.6	(3)
B lymphocytes, normal	1.83 ± 1.3	(6)
B lymphocytes, leukemic	4.93	(2)
Platelets	0.08	(2)

Note. Items in parentheses indicate the number of samples.

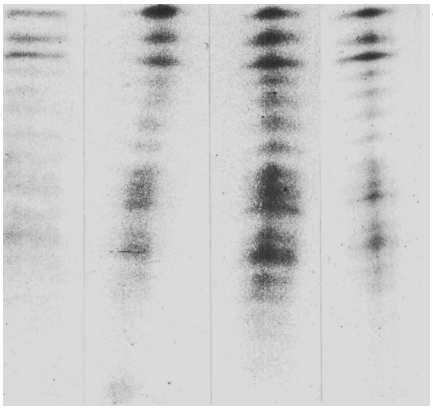

FIG. 2. Four replicate isoenzyme patterns of granulocytes from four experiments.

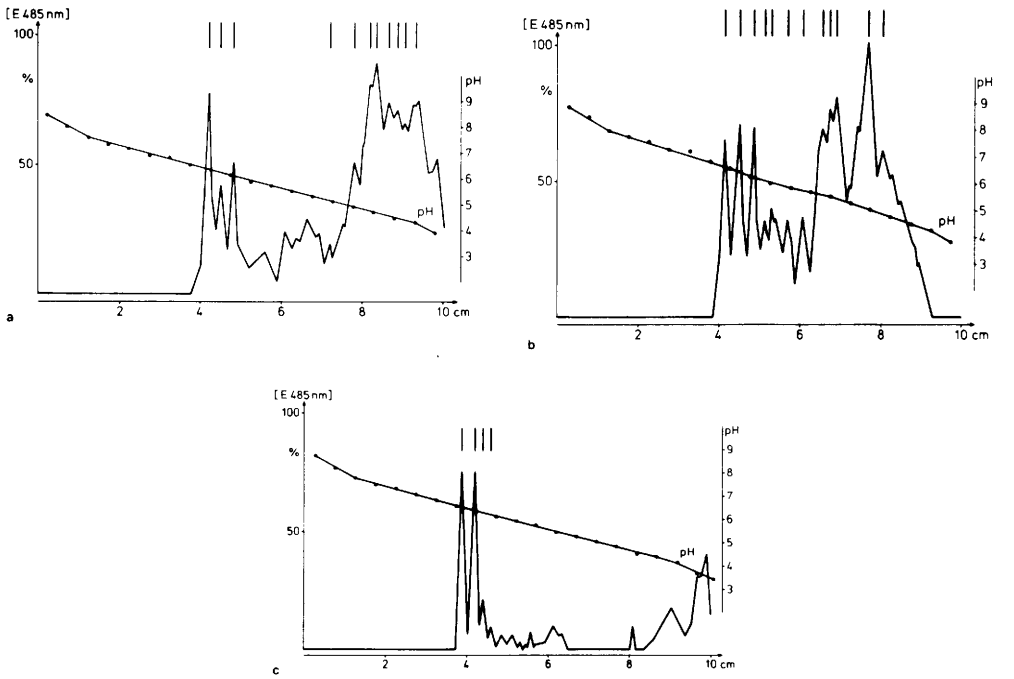

FIG. 3. Isoelectric focusing pattern of AcP extracted from monocytes (a), granulocytes (b), and platelets (c).

pattern identical to that of blood monocytes. The three cathodic bands, highly characteristic of the monocytic myeloid cell line, were of considerable intensity in all cases of monocytic leukemia (Fig. 5b).

Specimens collected from samples of T-cell leukemias showed a high intensity for the solitary cathodic band of T lymphocytes with a p*I* of 6.3. The minor activities seen between pH 5.0 and 4.0 could easily be attributed to contamination with monocytes (Fig. 5c). Like normal B lymphocytes, B-lymphocytic leukemias revealed, in addition to the cathodic band (p*I* 6.3), 11 more bands between pH 5.3 and 3.8. Thus there was a full accommodation of the IEF pattern of normal B lymphocytes with respect to that of neoplastic B lymphocytes (Fig. 5d).

Discussion. As a typical lysosomal enzyme (23) AcP is abundantly present in cells rich in lysosomes. Monocytes and their functional or neoplastic derivatives have correspondingly the highest enzyme activity. The high AcP content of the monocytic cell line, as compared with other blood cell types, is easily rendered by

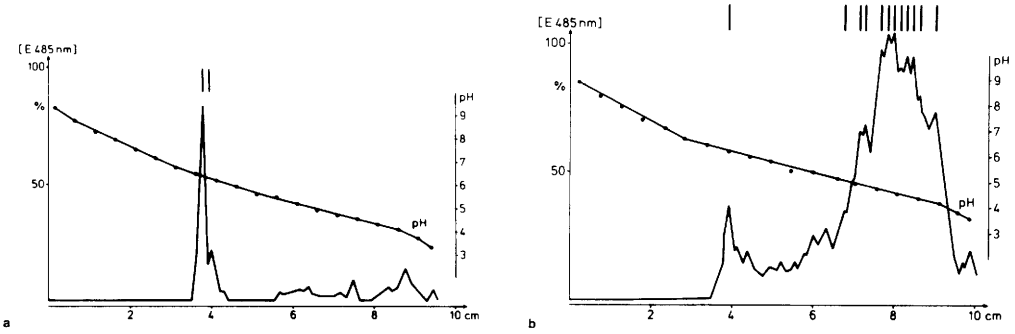

FIG. 4. Isoelectric focusing pattern of AcP extracted from T lymphocytes (a) and B lymphocytes (b).

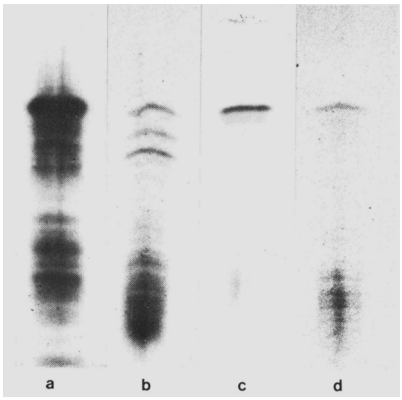

FIG. 5. AcP isoenzymes of AM (a), leukemic monocytes (b), and leukemic T (c) as well as leukemic B lymphocytes (d).

cytochemical as well as direct enzyme assay. Whereas neoplastic monocytes showed enzyme values within the range of normal monocytes, the exposure to inhalative agents seems to have induced an exorbitant amount of AcP in AM. Granulocytes showed the next highest AcP activity, again reflecting their copious lysosome content. B and T lymphocytes lacking phagocytic function have poorly developed lysosomal equipment and consequently showed extremely low enzyme activities under normal conditions as well as in their neoplastic variants (Table III). Despite these drawbacks, AcP have been widely applied in cytochemical tests to detect and classify normal lymphocytes and their neoplastic variants in different lymphoproliferative disorders (3, 4). The reason for this is the more or less cell-specific cytochemical pattern of enzyme activity in B and T lymphocytes. B lymphocytes, literally lacking clearly discernible lysosomes, show a faint diffuse enzyme activity, hardly detectable on cytochemical tests. T lymphocytes on the other hand exhibit dispersed or aggregated lysosomes as shown by electron microscopy. The cytochemical enzyme pattern apparently follows the same distribution, being evenly dispersed throughout the cytoplasm in about 10% of T lymphocytes (8) or concentrated in large granules representing aggregated lysosomes on the electron-microscopic level (24).

The growing information on the clonal diversification of T lymphocytes and especially the introduction of technical possibilities to identify and isolate T-lymphocyte subpopulations, e.g., T helper and suppressor cells (25), contributed significantly to the conceptual understanding and interpretation of the encountered cytochemical differences. Consequently the question arose whether or not the cytochemical heterogeneity of AcP in different blood cells was associated with a typical distribution pattern of isoenzymes.

Various attempts have been made to analyze the polymorphism of the lysosomal AcP. The results gained by polyacrylamide disc electrophoresis (26–28) were not convincing with respect to their cell specificity. For normal blood cells five isoenzyme loci could be distinguished on the basis of conventional electrophoresis. The most cathodal band, especially prominent in granulocytes and monocytes, was also found in thymocytes (28). On the other hand the third band, primarily lymphocyte specific, was detectable in platelets as well. These and other results show that conventional polyacrylamide electrophoresis with a maximum of five bands for at least five different blood cell types is hardly apt to provide isoenzyme patterns specific to the individual blood cell types.

The introduction of IEF brought about a considerable improvement of the resolution capacity, which was successfully used for the detection of enzyme variants (29). This also applies to the analysis of the AcP isoenzymes in different blood cell types (Figs. 3 and 4). A group of three bands within the pH range of 6.3 and 6.0 was shared by myelogenous cell lines, e.g., monocytes, granulocytes, and platelets. The most cathodal band with a p*I* value of 6.3 was detectable in all blood cell types investigated. T lymphocytes were the only cell line whose IEF pattern was confined to this solitary locus, whereas B lymphocytes were easily discernible by their additional 11 isoenzymes localized well apart between pH 5.3 and 3.8. Considering the individual isoenzyme patterns it is evident that each blood cell type is characterized on the basis of its cell-specific isoenzyme constellation.

On the other hand certain isoenzyme loci seemed to be shared by cytogenetically close-related cell types.

The cell-specific isoenzyme pattern of AcP proved to be fairly constant within the borders of technical variations, provided that identical net enzyme activities were applied (Fig. 2). Otherwise the possibility cannot be excluded that weak bands might escape identification. In addition, one must keep in mind the unavoidable shortcoming that in the direct enzyme assay a different substrate was used. Thus the stain intensity of the different bands does not necessarily reflect the photometrically measured values. This uncertainty has been met with to some extent by correlating activity values obtained using both methods (Fig. 1).

The constancy and reproducibility of the isoenzyme patterns allow the conclusion that such patterns might represent a reliable biochemical cell marker. In this case isoenzyme patterns might be useful, whenever identification or classification of a certain cell line could not be elaborated by conventional methods. This is best exemplified by considering functional derivatives or neoplastic variants of the different blood cells investigated. AM have repeatedly been shown to originate from bone-marrow-derived blood monocytes (30). The analysis of their AcP isoenzyme pattern confirms the close cytogenetic relationship between these two cell types. The accordance of the isoenzyme loci has often proved to be representative of a reliable means of population analysis (31) and of disclosing cytogenetic relationship (32–34). The results with leukemias investigated in the present study are well in line with this view. Not only did the monocytic leukemias show an isoenzyme pattern identical to that of normal blood monocytes, but also it was possible to distinguish T-cell leukemia from B-cell leukemia on the basis of the cell-specific isoenzyme pattern of the corresponding normal counterparts.

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