Studies on the Clinical Application of a Double-Stain Method for Peroxidase and Lysozyme Activities in Leukemic Cells

Takeshi KAGEOKA, M.D.

Third Department of Internal Medicine. Yamaguchi University School of Medicine, Ube City,755, Japan

(Received April 24, 1978)

INTRODUCTION

Cytochemical methods are being used routinely in the study of enzymes in leukemic cells, and have proved to be useful in the differential diagnosis of leukemia and differentiation of leukemic cells[1]. These methods provide the advantage of permitting the observer to study the clinical properties of individual cells. One such method, the peroxidase reaction, is a routine laboratory procedure widely used for identification of the myeloid and monocytic series of leukemic cells. Peroxidase is present in cells of the myeloid and monocytic series, and the enzyme activity increases with maturation of the cells. However, the peroxidase activity is stronger in the myeloid series than in the monocytic series[2]. Lymphocytic cells do not exhibit a peroxidase reaction, but there are also instances when cells of the myelocytic and monocytic series show a negative reaction[1,2]. The differential diagnosis of leukemic cells is particularly difficult if they are too immature morphologically to produce peroxidase enzymes.

Lysozyme activity in the myeloid and monocytic series can be observed by the cytochemical method, and the activity increases with maturation[3]. Normally, enzyme activity is noted at the progranulocyte stage and is strongest in polymorphonuclear cells. Monocytes also have strong lysozyme activity[3]. This activity is not observed in lymphocytes or normal myeloblasts, but it is sometimes noted in blast cells in cases of myelocytic and monocytic leukemia[3,4]. The serum and urine levels of lysozyme are elevated in acute leukemia, a highly valuable characteristic from the standpoint of establishing a diagnosis, but there are occasional cases of myelocytic and myelomonocytic leukemia in which the levels remain normal[5,6,7]. It is thought that serum and urinary lysozyme activity reflect more the total mass of leukemic cells containing lysozyme than any special cell type[8]. Therefore, fluctuation in these levels may be observed during the course of treatment.

Neither peroxidase nor lysozyme activity can be observed in lymphocytic cells, however with the exception of some blast cells, both enzyme activities are present in the myeloid and monocytic series. The peroxidase activity is stronger in the myeloid series than the monocytic series, while the lysozyme activity is stronger during the early stage in the monocytic series than the myeloid series[4]. In the differential diagnosis of acute leukemia using routine hematological procedures, it is often difficult to determine morphologically whether it is myelocytic or myelomonocytic leukemia[9]. In such instances, it might be possible to obtain more information useful for differential diagnosis if one leukemic cell could be stained for two types of enzymes simultaneously. Double staining of lysozyme and peroxidase was attempted by Syrén and Raeste [10], but morphological detail was not as clear compared with separate staining. Catovsky and Galton also reported a method for simultaneous cytochemical staining, but they did not mention the technical details or make any evaluation as to practical application[4]. This report describes a method for simultaneous demonstration of peroxidase and lysozyme which is simple and can be performed routinely in the hematological evaluation of acute leukemia. The results of its application to various types of acute leukemia as well as findings of a comparative study of the serum and urinary lysozyme activity values in cases in which samples had been collected at the time of double staining are also presented.

MATERIALS AND METHOD

Eighteen patients with acute leukemia hospitalized on the ward of the Third Department of Internal Medicine, Yamaguchi University Hospital were studied. A portion of the blood and bone marrow aspirate was used to prepare specimens for differential cell count and the remainder was used for double staining for peroxidase and lysozyme activity. The hematological diagnosis of patients was made by hematologists not associated with this study employing the usual clinical, hematological and morphological criteria. The diagnoses were as follows: acute lymphocytic leukemia, 4; acute progranulocytic leukemia, 2; acute myeloblastic leukemia, 6; acute myelomonocytic leukemia, 4; and erythroleukemia, 2.

McJunkin's cytochemical method[11] for peroxidase and the cytochemical method of Syrén and Raeste[10] for lysozyme were modified as follows for double staining: A 100 μl amount of heparinized blood or bone marrow aspirate was mixed with 100 μl of a freshly suspended bacteria solution (dried Micrococcus lysodeicticus, 120 mg in 1 ml of

6% hydroxyethyl starch in saline solution), cooled to 4°C and agitated gently for 5 seconds. The mixture was immediately smeared on a microscopic glass slide and air dried, following which it was fixed in a 1:3 mixture of 10% neutral formalin and 96% ethanol cooled to 4°C for 30 seconds. Following brief rinsing at 4°C with 0.01 M phosphate buffer, pH 6.2, the peroxidase staining solution (0.5 g benzidine and 0.15 ml hydrogen peroxide in 200 ml distilled water) was poured onto the smear and left for 2 minutes at room temperature for the peroxidase reaction. Immediately after completion of the reaction, the smear was rinsed with the same phosphate buffer and incubated in 0.01 M phosphate buffer, pH 6.2 at 37°C for 15 minutes for the lysozyme reaction. The smear was then immersed in 3% hydroxyethyl starch in 0.01 M phosphate buffer, pH 6.2, dried by cooled air, and counterstained with Wrights' stain.

The above process makes it possible to observe each cell for both lysozyme and peroxidase activity. When the lysozyme activity is positive, an area of bacterial lysis or weak staining of the bacteria can be seen immediately adjacent to the cell, while positive peroxidase activity can be seen as varying degrees of yellowish-brown pigmentation in the cytoplasm, as shown in Figure 1. At the same time, double staining of a normal blood sample was also examined as a control to check the lysozyme and peroxidase activities in the neutrophils and monocytes.

The serum and urinary lysozyme activity was mesured by the method of Smolelis and Hartsell[12] using Micrococcus lysodeicticus and crystalline lysozyme as standard. Lysozyme activity was expressed as micrograms per milliliter serum and urine. The urine sample which was collected for 24 hours was used for lysozyme assay of urine.

Micrococcus lysodeicticus and crystalline lysozyme were obtained commercially from Sigma, United States and hydroxyethyl starch from Morishita Seiyaku, Japan. The other reagents used were of analytical grade. Determination of lysozyme activity was carried out by a Gilford Model 2500 Spectrophotometer.

RESULTS

The presence of lysozyme activity was determined by the bacterial lysis of micrococcus in the vicinity of the cell, and the strength was graded as follows by the extent of lysis: L_0, no change observed in the bacteria immediately adjacent to the cell nor in their morphologic characteristics; L_1, slight lysis noted only in bacteria immediately adjacent to the cell; L_2, narrow ring of lysis surrounding the cell; L_3, forma-

Fig. 1.

Demonstration of peroxidase and lysozyme activity in the same leukemic cells from cases of myelomonocytic leukemia (A,B,C,D,E,F,G,H), myeloblastic leukemia (I) and lymphocytic leukemia (J).

A. Atypical monocytoid cell, showing strongly positive lysozyme activity with bacterial lysis around the cell (L_3) and negative peroxidase (P_0).

B. Immature leukemic cells, showing a strongly positive peroxidase reaction in the cytoplasm (P_3) and negative lysozyme activity (L_0).

C. Immature leukemic cell, showing a strongly positive peroxidase reaction with yellow-brown granules throughout the cytoplasm (P_4) and moderately positive lysozyme activity (L_2).

D. Atypical monocytoid cell, showing a slightly positive peroxidase reaction in the cytoplasm (P_1) and moderately positive lysozyme activity (L_2).

E. Atypical monocytoid cell, showing a slightly positive peroxidase reaction in the cytoplasm (P_1) and moderately positive lysozyme activity (L_2).

F. Immature leukemic cell, showing a strongly positive peroxidase reaction with yellow-brown granules throughout the cytoplasm (P_4) and moderately positive lysozyme activity (L_2).

G. Atypical monocytoid cell, showing a moderately positive peroxidase reaction in the cytoplasm (P_2-P_3) and strongly positive lysozyme activity (L_3).

H. Atypical monocytoid cell, showing a negative peroxidase reaction (P_0) and slight lysozyme activity (L_1-L_2).

I. Immature leukemic cells, showing negative lysozyme activity (L_0) and a moderately positive peroxidase reaction (P_2) except for one (P_0).

J. Immature lymphocytic cells, showing no peroxidase or lysozyme activity (P_0, L_0). A neutrophil with positive peroxidase and lysozyme activity can be seen among the cells.

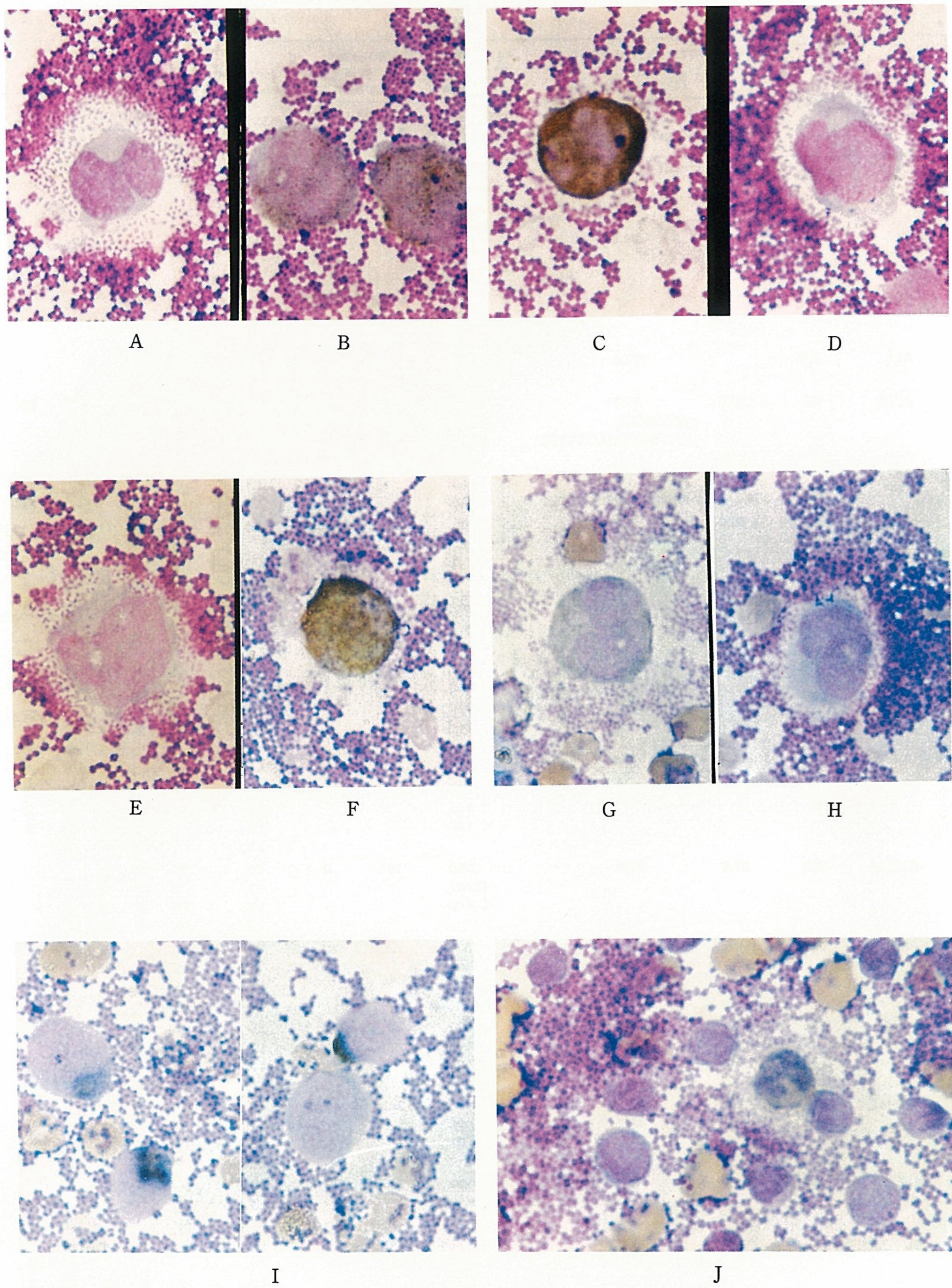


Fig. 1.

Table 1 Serum and urinary lysozyme activity and correlaton of lysozyme and peroxidase activity in leukemic cells (% of leukemic cell)

CASE	DIAGNOSIS	SAMPLE	DIFFERENTIAL CELL COUNT OF THE SAMPLE (%)		PERIPHERAL BLOOD WBC (cells/mm^3)	LYSOZYME ACTIVITY (μg/ml)		CYTOCHEMICAL DEMONSTRATION					
			BLASTS	IMMATURE ATYPICAL CELLS	(BLASTS %) <ATYPICAL CELLS %>	SERUM 4.0-9.0 (NORMAL)	URINE 0 (NORMAL)	LYSOZYME ACTIVITY	PEROXIDASE ACTIVITY P0	P1	P2	P3	P4
1	ALL	BM	79.5	2.7	11200 (69.5) < 2.5>	7.1	1.8	L0	100				
2	ALL	BM		99.0	21100 (67.5) < 2.5>	7.7	–	L0	100				
3	ALL	BM	6.6	4.4	530000 (84.0) <12.5>	5.1	0	L0	100				
4	ALL	BM		92.5	97700 <85.0>	2.9	–	L0	100				
5	APGL	PB	40.0	47.0 ATYPICAL PROGRANULOCYTES	21300 (40.0) <47.0>	–	–	L0				85	13
								L1				2	
6	APGL	BM	2.5	79.7 ATYPICAL PROGRANULOCYTES	2000 (2.0) <65.0>	15.7	0	L0				1	77
								L1					12
								L2					10
7	AML	PB	45.5	15.0	44000 (45.5) <15.0>	36.2	126.0	L0	82	7	11		
8	AML	BM	65.8	3.6	–	–	–	L0	1	1	62	36	
9	AML	PB	34.5	7.0	52700 (34.5) < 7.0>	46.8	157.0	L0	4	2	45	46	3
10	AML	BM	33.6	17.3	400 (0.0) < 1.0>	–	–	L0	32	10	30	24	
								L1			2	2	
11	AML	BM	26.4	17.4	47000 (8.0) < 9.5>	–	–	L0	37	6	14	16	8
								L1			2	3	2
								L2			4	4	4
12	AML	BM	22.0	29.7	1500 (4.0) < 1.0>	21.7	1.0	L0			2	42	7
								L1	1		1	16	2
								L2			4	17	2
								L3			4	2	
13	AMMoL	BM	37.5	35.0	3200 (32.0) < 5.0>	10	0	L0	40	3	2		
								L1	2	2	2		
								L2	11	5	5	4	
								L3	5	8	11		
14	AMMoL	BM	20.5	26.2	7300 (6.0) < 8.0>	63.6	1.1	L0	36		9	1	
								L1	4		2		1
								L2	11	2	4	10	
								L3	10	1	4	5	
15	AMMoL	BM	3.0	4.6	2100 (1.0) < 3.0>	22	22.5	L0	27	5	8	5	
								L1	5	2	8	2	
								L2	11	6	14	3	
								L3	2	1	1		
16	AMMoL	PB	27.0	4.0	2300 (27.0) < 4.0>	34.7	408.0	L0	40	1	1		
								L1	4	4	2		
								L2	5	5	20	5	
								L3	6	1	3	3	
17	EL	PB	41.0	0	15100 (41.0)	24.7	5.7	L0	30	3	2	29	28
								L1	2			1	1
								L2				2	2
18	EL	BM	1.0	28.4	1300 < 3.0>	19.7	0	L0			1	6	7
								L1				4	3
								L2	3	3	10	8	3
								L3	11	15	13	12	1

PB = PERIPHERAL BLOOD, BM = BONE MARROW, ALL = ACUTE LYMPHOCYTIC LEUKEMIA, APGL = ACUTE PROGRANULOCYTIC LEUKEMIA, AML = ACUTE MYELOBLASTIC LEUKEMIA, AMMoL = ACUTE MYELOMONOCYTIC LEUKEMIA, EL = ERYTHROLEUKEMIA.

tion of a broad zone of lysis.

The peroxidase reaction was detected by the presence of yellow-brown pigmentation in the cytoplasm, and there was a wide range in the extent of the reaction which was classified as follows: P_0, no pigmentation of the cytoplasm observed; P_1, faint localized pigmentation in the cytoplasm; P_2, localized areas of yellowish-brown granules; P_3, yellowish-brown granules scattered throughout the cytoplasm; P_4, coarse yellowish-brown granules scattered throughout the cytoplasm and also covering the nucleus, rendering its shape indistinct.

The leukemic cells used for double staining were defined as unclassified blast cells, atypical progranulocytes, atypical monocytes, atypical myelocytes, atypical monocytoid cells and atypical lymphoblasts. One hundred cells were observed and classified according to the degree of activity.

The normal range of lysozyme activity which was measured by the method of Smolelis and Hartsell[12] was 4.0–9.0 μg/ml in serum, but was

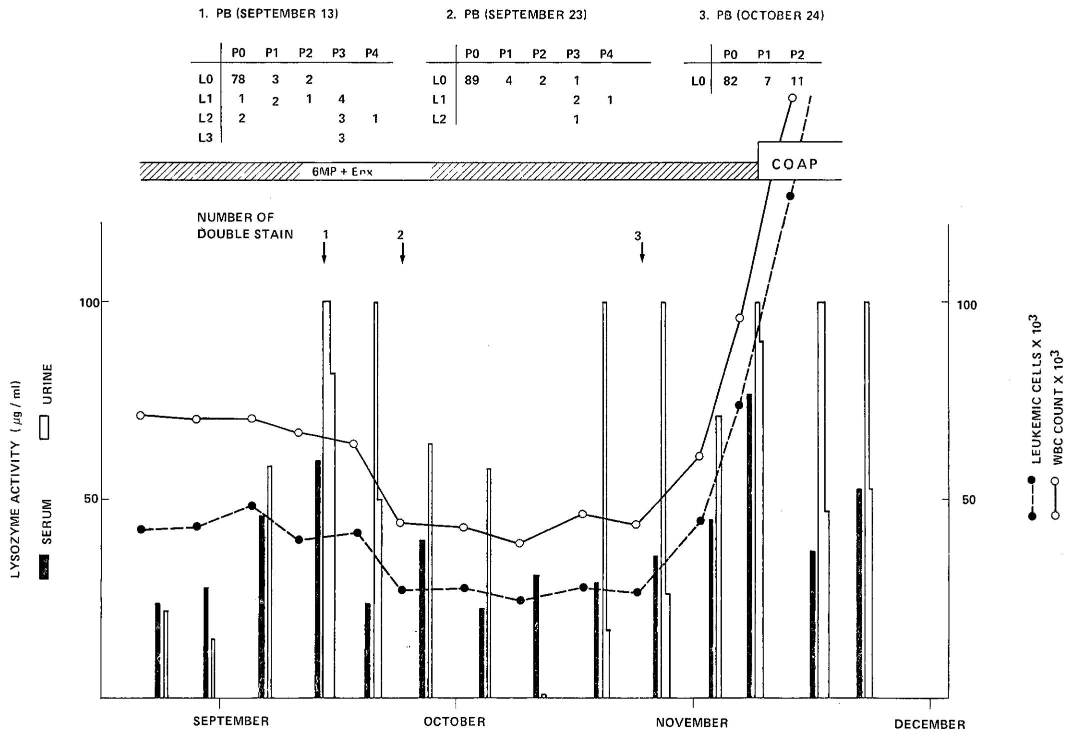
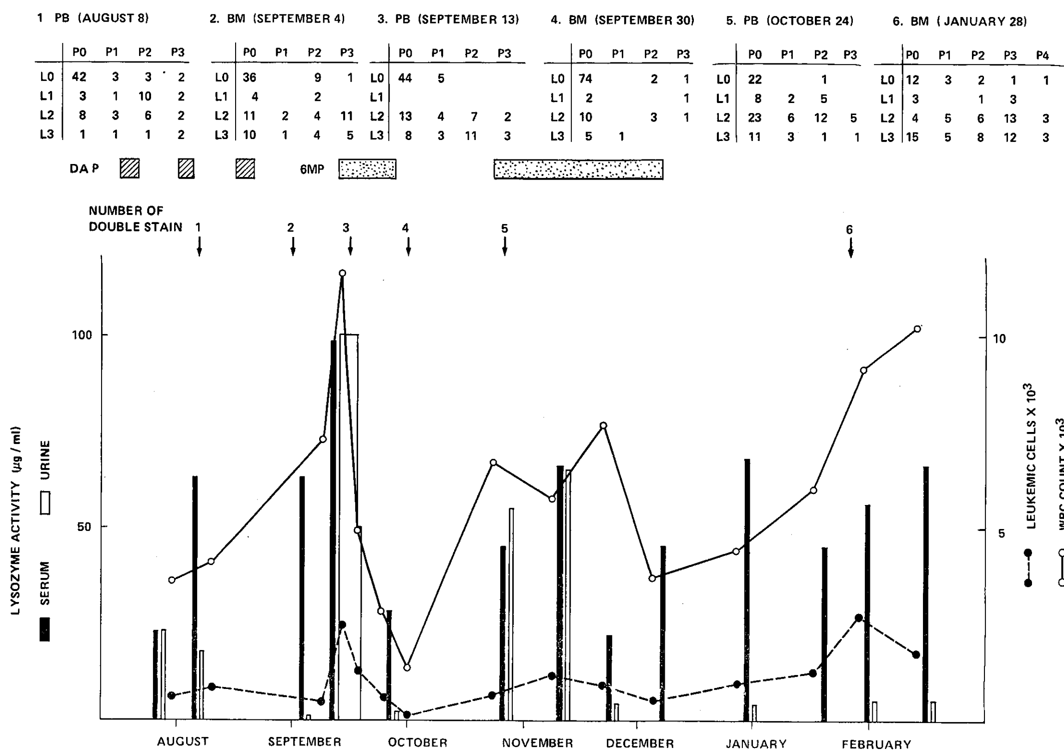


Fig. 2. The changes in serum and urinary lysozyme activity and results of double stain in a patient with acute myeloblastic leukemia in relation to clinical course.



1 PB (AUGUST 8)

	P0	P1	P2	P3
L0	42	3	3	2
L1	3	1	10	2
L2	8	3	6	2
L3	1	1	1	2

2. BM (SEPTEMBER 4)

	P0	P1	P2	P3
L0	36		9	1
L1	4		2	
L2	11	2	4	11
L3	10	1	4	5

3. PB (SEPTEMBER 13)

	P0	P1	P2	P3
L0	44	5		
L1				
L2	13	4	7	2
L3	8	3	11	3

4. BM (SEPTEMBER 30)

	P0	P1	P2	P3
L0	74		2	1
L1	2			1
L2	10		3	1
L3	5	1		

5. PB (OCTOBER 24)

	P0	P1	P2	P3
L0	22		1	
L1	8	2	5	
L2	23	6	12	5
L3	11	3	1	1

6. BM (JANUARY 28)

	P0	P1	P2	P3	P4
L0	12	3	2	1	1
L1	3		1	3	
L2	4	5	6	13	3
L3	15	5	8	12	3

Fig 3. The changes in serum and urinary lysozyme activity and results of double stain in a patient with acute myelomonocytic leukemia in relation to clinical course.

normally not detected in urine. The test results are shown in Table 1.

The clinical course of one case each of acute myeloblastic leukemia and acute myelomonocytic leuekmia as well as the changes in serum and urinary lysozyme activity, findings in the peripheral blood and cytochemical lysozyme and peroxidase activity are shown in Figures 2 and 3. As the patient with acute myeloblastic leukemia was of advanced age, aggressive combination chemotherapy was not administered. The leukocyte count fluctuated slightly at about 50,000/mm^3. Both the serum and urinary lysozyme activities changed greatly during the course of treatment, but the double stain method continued to demonstrate the characteristic findings of this entity (Figure 2). There was good correlation between the changes in leukocyte counts and serum and urinary lysozyme activity in relation to time in these cases. Accordingly, serum and urinary lysozyme activity changed greatly during the course of treatment. However, the characteristic cellular morphologic features in this disease could always be demonstrated by double staining (Figures 2 and 3).

DISCUSSION

The use of hydroxyethyl starch instead of physiological saline made it possible to prevent cell smudging, nuclear swelling and loss of cells and bacteria from the preparation. Comparison of the lysozyme activity of the cytochemical stain prepared by suspension of bacteria in physiological saline versus hydroxyethyl starch showed that the latter presented a slightly narrower band. Further, study of cell morphology in the specimens revealed that many cells with poorly defined margins were present on the slides prepared with physiological saline. Such cells had a wider lysed band around them than did those of the same stage of maturation but with normal morphology. It is presumed that the wider band is caused by escape of enzyme from the cytoplasm due to destruction of the cell. When hydroxyethyl starch is used, normal cell morphology is retained. Therefore, it seems more reasonable to assume that the width of the lysed band changes in proportion to the volume of enzyme in the cell and thus it is felt to be an appropriate index for determination of lysozyme activity.

Our method, in which the peroxidase reaction precedes the lysozyme reaction, provides a well-demarcated cell margin and a clearer zone of lysis than does the method of Syrén and Raeste[10], where reaction of the two enzymes takes place in the same buffer solution, or does the method of Catovsky and Galton[4], where peroxidase staining is performed after the lysozyme reaction. The sequence in our method precludes brown staining of the zone of lysis which enables easy evaluation of the enzyme activity.

Despite the fact that there were a large number of leukemic cells in the peripheral blood of the acute lymphocytic leukemia cases, the serum and urinary lysozyme activities were low. Further, double staining of the leuekmic cells failed to demonstrate either lysozyme or peroxidase activity. There were only 2 cases of acute progranulocytic leukemia, and a slight increase in serum lysozyme activity was noted in one, but there was no reaction in the urine. Acute progranulocytic leukemia is a subtype of acute myeloblastic leukemia, and is morphologically more differentiated. The leukemic cells of acute progranulocytic leukemia also possess extremely strong peroxidase activity which can be specifically stained by the double stain technique, and thus distinguished from myeloblastic leukemia. In one of the two cases, many peroxidase positive leukemic cells showed lysozyme activity, which may serve to suggest there is a difference by case in the degree of differentiation (Cases 5 and 6). In the cases with acute myeloblastic leukemia, there was a

marked increase in the leukocyte count in the peripheral blood as well as an increase in serum and urinary lysozyme activity, but the rate of increase in the latter was not as great as that of the increase in number of immature cells. The cases could be classified into three types based on results of double staining: Type 1, a case with leukemic cells that were negative for both peroxidase and lysozyme activity (Case 7); Type 2, cases with cells that have a wide range of peroxidase activity from weak to strong, but hardly any lysozyme activity (Cases 8-10), and type 3, cases with numerous leukemic cells which are positive for both peroxidase and lysozyme activity (Cases 11 and 12). When the leukemic stem cells differentiate in the direction of myeloblast to promyelocyte, it is thought that peroxidase activity can be demonstrated earlier than lysozyme activity[4]. Therefore, a simultaneous positive reaction of the two enzymes indicates a more advanced stage of cellular differentiation[4]. Thus, leukemic cells which fail to demonstrate any difference in degree of differentiation morphologically, may be distinguished by degree of enzyme differentiation in some cases or even in the same individual. However, we were unable to ascertain whether the degree of differentiation in these leukemic cells changed during the course of the disease. In the course of maturation of monocytes, lysozyme activity is predominant over that of peroxidase. Study of the cases with acute myelomonocytic leukemia (Cases 13-16) showed that there was a decrease in number of peripheral blood leukocytes because they had all been under chemotherapy, and the degree of serum and urinary lysozyme activity was not markedly higher than that of the other types of acute leukemia. However, when the degree per cell was calculated, it proved to be much higher than the others. Among the acute myelomonocytic leukemia cases with characteristic findings of lysozyme positive and peroxidase negative cells, many cells demonstrating positive activity for both enzymes were found. Monocytes are considered to arise from bone marrow myeloblast[13], and as indicated by the term "acute myelomonocytic leukemia" are thought to possess the morphological characteristics of both myeloid and monocytoid cells. The presence of such a cell line was confirmed by this cytochemical method. The characteristic finding of lysozyme positive and peroxidase negative leukemic cells in acute myelomonocytic leukemia is thought to indicate that these cells mature in the monocytic direction. Unless the results of the double staining method specific for myeloblastic leukemia and acute myelomonocytic leukemia are maintained throughout the course of the disease, their diagnostic value is reduced to one half. The peripheral leukocyte count, serum and urinary lysozyme activity and double stain

findings of lysozyme and peroxidase activity were followed in one case each of acute myeloblastic leukemia and acute myelomonocytic leukemia. In both cases, the fluctuation of leukocyte counts was reflected in the serum and urinary lysozyme values which changed greatly. However, the activities of both enzymes as demonstrated by the double staining method maintained their own characteristic features throughout the course, and the two could readily be distinguished (Figures 2 and 3). In erythroleukemia, generally two phases were observed: an early erythroblastic predominant phase and a late myeloblastic predominant phase[14]. The two cases presented in this report belong to the late phase category. Case 17 presented an acute myeloblastic leukemia pattern with practically all of the leukemic cells having peroxidase positive and lysozyme negative activity. On the other hand, in Case 18 many of the cells demonstrated lysozyme positive and peroxidase negative activity, presenting an acute myelomonocytic leukemia pattern. These findings seem to suggest that leukemic cells in the late phase of erythroleukemia differentiate in both the myeloid and monocytic directions.

In the acute leukemia cases reported here, we did not find a good correlation between serum and urinary lysozyme levels and type of leukemia. Further, there was a wide range of fluctuation in these values during the course of the disease in the same individual, and hence these findings were not considered adequate for the purpose of differential diagnosis. Karle and his associates[8] state that the plasma and urinary lysozyme values reflect the total leukemic lysozyme positive cell mass rather than a special cell type. It is considered that through application of this double staining method, lysozyme and peroxidase activities in the same leukemic cell will present a characteristic pattern according to the type of acute leukemia, and the specificity of the activity can be maintained without being greatly affected by changes in the state of the disease. Therefore, it is felt this method is useful in the differential diagnosis of acute leukemia, particularly acute myeloblastic and myelomonocytic leukemias.

SUMMARY

A simple double stain method for peroxidase and lysozyme (muramidase) activities in individual cells was devised and applied to the assessment of cellular differentiation and follow-up of the clinical course in acute leukemia. The results revealed characteristic patterns specific for the different types of acute leukemia, and the specificity of the patterns were maintained without being greatly affected by changes in the state of the disease.

The method was useful in differentiating acute myelomonocytic leukemia having numerous leukemic cells with lysozyme positive-peroxidase negative activity from acute myeloid leukemia with leukemic cells characterized by peroxidase positive-lysozyme negative activity.

ACKNOWLEDGMENT

The author is greatly indebted to Dr. Shiro Miwa, Professor, for his guidance throughout the study and wishes to express appreciation to his colleagues in the Third Department of Internal Medicine as well as to the technical staff of the department. A part of this study was published in the American Journal of Clinical Pathology, 67 : 481-484, 1977.

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