Lysozyme activity and nitroblue-tetrazolium reduction in leukaemic cells

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SYNOPSIS The cytochemical methods for lysozyme and nitroblue-tetrazolium reduction have been used to study the blast cells of acute myeloid leukaemia. Both proved useful in characterizing the cases with predominant monocytic differentiation.

The demonstration of lysozyme activity helped to define two main groups: (a) with predominantly lysozyme-negative cells (myeloblastic-promyelocytic), and (b) with considerable numbers of positive cells (monoblastic-monocytic). In addition this test was also of value in the differentiation of other leukaemic disorders. Reduction of nitroblue-tetrazolium was also a feature of monocytic differentiation. The combination of these two methods with those for myeloperoxidase and non-specific esterase activity contributes to the cytological characterization of acute myeloid leukaemia.

Numerous reports have emphasized in recent years the value of the serum and urine lysozyme (muramidase) estimations in the identification of the various morphological types of acute myeloid leukaemia (Osserman and Lawlor, 1966; Perillie, Kaplan, Lefkowitz, Rogaway, and Finch, 1968; Wiernik and Serpick, 1969; Ohta and Nagase, 1971; Catovsky, Galton, and Griffin, 1971). The evidence suggests that leukaemic cells with monocytic differentiation are the main source of lysozyme. Other minor sources of lysozyme present in the myeloid leukaemias include the neutrophils. The quantitative estimation of lysozyme in serum and urine provides no indication of the cellular source from which the enzyme is derived; the quantitative results would be much more useful if it were possible to identify the cells producing the enzyme in each case. A method for doing so has been available for many years (Briggs, Perillie, and Finch, 1966) and it is surprising that it has been so little used. We have used a modification of the method (Syrén and Raeste, 1971) which shows the lysozyme activity of leukaemic cells whose morphology is well preserved.

The capacity of normal mature neutrophils to reduce nitroblue-tetrazolium (NBT) *in vitro* when their oxidative metabolism is stimulated by bacterial products or phagocytosis has been extensively studied (Park, Fikrig, and Smithwick, 1968; Nathan, Baehner, and Weaver, 1969; Park and Good, 1970).

During the course of a study of the NBT reduction of neutrophils from patients with acute myeloid leukaemia (Goldman and Catovsky, 1972) it became apparent that a proportion of immature and mature monocytes from those cases could also reduce NBT. We have therefore used this test to see if it would help in the cytochemical analysis of acute myeloid leukaemia. In this paper we are not concerned with the reaction of mature neutrophils.

A preliminary report of this study has been published (Catovsky and Galton, 1972).

Materials and Methods

Material from 36 patients with the following diagnoses was studied: acute myeloid leukaemia 26; chronic granulocytic leukaemia 3; leukaemic reticulo-endotheliosis 2; chronic monocytic leukaemia 3; and malignant lymphoma in a terminal leukaemic phase (one reticulum-cell sarcoma and one poorly differentiated lymphocytic). Cases of acute myeloid leukaemia were classified according to morphological and cytochemical criteria (Catovsky *et al*, 1971) as myeloblastic, myelomonocytic, and monoblastic-monocytic. In addition, myelomonocytic cases were subdivided into types I and II according to the lysozyme concentrations and the degree of extramedullary disease as evidenced by gum hypertrophy, lymphadenopathy, and splenomegaly (Catovsky, Galton, and Robinson, 1972a). The type II cases were those in which the monocytic com-

ponent was dominant, whereas the granulocytic component was dominant in type I cases.

Lysozyme activity was demonstrated in the leukaemic cells by the cytochemical method of Syrén and Raeste (1971). Samples of peripheral blood from all the patients and of bone marrow from eight were collected in sequestrene. The test was performed within the first hour after collection, and a fresh suspension of *Micrococcus lysodeikticus* (Difco) in saline was used as substrate. Numerous smears were made after mixing for a few seconds equal parts of the bacterial suspension and the blood (using preferentially the leucocyte-rich plasma layer). The films were stained with May-Grünwald Giemsa and, in some, the myeloperoxidase reaction was also performed as in Dacie and Lewis (1968). Serum lysozyme was estimated in all the cases by means of the turbidimetric method of Parry, Chandan, and Shahani (1965) against standards of egg-white lysozyme (Seravac). Normal concentrations in our laboratory are in the range of 4 to 9 µg/ml.

Nitroblue-tetrazolium (NBT) reduction before and after incubating the blood for 10 minutes with *E. coli* endotoxin (NBT 'stimulated', Park and Good, 1970) was studied by the method of Park *et al* (1968) in blood samples collected in lithium heparin from 17 patients. For the assessment of our results we have considered separately the reaction in the immature leukaemic cells from that of the mature neutrophils. The effect of potassium cyanide at a concentration of 1 mM/ml on the NBT reduction was measured in three myelomonocytic (II) cases. The NBT reaction was also studied in macrophages derived from leukaemic monocytes after three, five, and seven days of culture in Leighton tubes. The cell cultures were stimulated either with *E. coli* endotoxin or a live suspension of *Candida albicans*.

The cytochemical method of Wachstein and Wolf (1958) for non-specific esterases with and without simultaneous incubation with sodium fluoride (Schmalzl and Braunsteiner, 1968) was performed in the blood and bone-marrow films of nine cases of acute myeloid leukaemia, one of leukaemic reticuloendotheliosis, and one of reticulum-cell sarcoma in the terminal leukaemic phase.

Results

LYSOZYME ACTIVITY IN LEUKAEMIC CELLS

The preservation of the cell morphology in preparations of the cytochemical method was very good, although azurophilic granulation was often indistinct. Positive lysozyme activity was seen as an area of bacterial lysis in the vicinity of the cell; in cells with moderate activity the area of lysis was small and it was absent around cells with no activity. No attempt will be made here to separate intermediate degrees of reaction. In myeloblastic leukaemia the blast cells usually did not show any evidence of activity (Fig. 1, A); very occasionally a positive cell was found. The proportion of positive cells found in these cases is given in the Table. Myelomonocytic cases varied in their proportion of positive cells: those of type I showed two distinct populations of blast cells, positive and negative (Fig. 1, B, C), the negative ones predominating (Table). The positive blasts were usually large monocytoid forms, while the negative ones were often smaller and had a round nucleus. In type II (Fig. 2, A) as well as in monoblastic-monocytic cases (Fig. 2, B, C) a high proportion of the leukaemic cells was positive (Table). In one case, predominantly monoblastic, only 10% of the cells were positive. In the three cases of chronic monocytic leukaemia the proportion of positive monocytoid cells was very high (90 to 100%). In chronic granulocytic leukaemia all the cells of the granulocytic series, from myelocytes to polymorphonuclear neutrophils were positive, but it was difficult to identify positive promyelocytes (as in acute myeloid leukaemia or chronic monocytic leukaemia). A marked positive reaction was seen in nearly all the malignant cells in the case of reticulum-cell sarcoma in the leukaemic phase (Fig. 3, B). The cells were large, poorly differentiated blast cells with a very fine cytoplasmic granulation. No lysozyme activity was demonstrated in the blast cells in the case of poorly differentiated (lymphocytic) lymphoma in the leukaemic phase (Fig. 3, C) or in the two cases of leukaemic reticuloendotheliosis (Fig. 3, A).

There was a positive correlation between the proportion of positive cells and the serum lysozyme

Type of Acute Myeloid Leukaemia	No. of Cases	Lysozyme-positive Cells (%) ¹		Serum Lysozyme (µg/ml)	No. of Cases	NBT-positive Cells (%) ¹	
		Range	Average			Range	Average
AMbL	10	0-0.5	0.1	2.5-9	5	0-1.0	0.5
AMML (I) ^a	8	4-46	22.5	2.6-55	4	0-13	6.7
AMML (II)	5	42-95	77	43-230	4	15-45	33.3
AMoL	3	10-90	61	49-290	2	6-56	31

Table Proportion of lysozyme and NBT-positive cells in the various types of acute myeloid leukaemia

AMbL = myeloblastic; AMML = myelomonocytic; AMoL = monocytic. ¹Leukaemic cells, excluding mature neutrophils

^aFor explanation of I and II see text

Fig. 1A.

Fig. 1 *Cytobacterial demonstration of lysozyme activity*

A AMbL, bone-marrow film; blast cells lacking lysozyme activity, the bacteria are seen intact around the cells ($\times 750$)

B and C AMML (I), peripheral blood film, each showing a positive and a negative blast cell. Positive cells are surrounded by an area of bacterial lysis. Lysed organisms appeared paler and further apart from each other in this and subsequent photographs ($\times 1500$).

Fig. 1B.

Fig. 1C.

A

Fig. 2 *Cytobacterial demonstration of lysozyme activity.*

A *AMML (II), peripheral blood film showing a high proportion of positive blast cells (× 750).*

B and C *AMoL, blast cells with monocytic morphology and positive bacterial lysis (× 1500).*

B

C

A

B

C

Fig. 3 *Cytobacterial demonstration of lysozyme activity.*

A *Negative reaction in bone-marrow cells from a case of leukaemic reticuloendotheliosis ($\times 1500$)*

B *Positive bacterial lysis in a blast cell from a reticulum-cell sarcoma in terminal leukaemic phase ($\times 1200$).*

C *Negative reaction in bone-marrow cells from a poorly differentiated lymphocytic lymphoma ($\times 1500$)*

concentrations in acute myeloid leukaemia (Table). Furthermore, a statistically significant correlation between the absolute numbers of circulating positive cells and the serum concentrations of lysozyme (Fig. 4) was found when cases of acute myeloid and chronic monocytic leukaemia were grouped together (correlation coefficient $r = 0.75$, $P < 0.001$).

Simultaneous staining of the lysozyme preparations for myeloperoxidase showed: (1) myeloblasts positive for myeloperoxidase usually lacked lysozyme activity; the same pattern was seen in a case in which the majority of leukaemic cells were promyelocytes; (2) in myelomonocytic leukaemia some lysozyme-positive blasts were also positive for myeloperoxidase; (3) in monoblastic-monocytic and chronic monocytic leukaemia the cells were usually negative for myeloperoxidase and positive for lysozyme activity; (4) in cases of undifferentiated blast-cell leukaemia both reactions were negative; one of these cases was included in our myeloblastic group.

Results with the non-specific esterase reaction were compared with those of the cytochemical test for lysozyme activity in 11 cases. It was weak or moderately positive in the majority of blast cells of acute myeloblastic leukaemia and myelomonocytic leukaemia (I), but a few strongly positive cells were found in myelomonocytic leukaemia type I. A higher proportion of cells were strongly positive in the type II cases and in those of monoblastic-monocytic leukaemia and in the case of reticulum-cell sarcoma in the leukaemic phase. In all cases with a strong

positive reaction for non-specific esterase the activity was abolished by simultaneous incubation with NaF.

In general, the proportion of cells with a strong reaction corresponded to the number of lysozyme-positive cells. For example, in the case of monoblastic monocytic leukaemia with only 10% of lysozyme-positive blasts, approximately 10% of the cells were strongly positive with the esterase reaction, the rest being only moderately positive. A weak reaction, slightly inhibited by NaF, was found in a case of leukaemic reticuloendotheliosis.

NITROBLUE-TETRAZOLIUM REDUCTION

In addition to the NBT reaction which occurs in the neutrophils, a positive reaction was also noted in normal as well as in leukaemic monocytes. As in the neutrophils, positively reacting monocytes contained a black cytoplasmic deposit of reduced NBT (formazan). The reaction seemed to occur more readily in cells which appeared slightly damaged in the final films or agglutinated in groups of four or five. The preservation of the cell morphology was not as good as in the method for lysozyme, and accurate counting was more difficult.

In myeloblastic leukaemia the majority of blasts were negative (Table). In myelomonocytic variable proportions of NBT-positive cells were present (Fig. 5, A, B) more often in type II (Table), which has a more conspicuous monocytic component. In acute and chronic monocytic leukaemia approximately 25% of the leukaemic cells were positive.

Fig. 4 Correlation between serum lysozyme and number of circulating lysozyme-positive cells in AML and CMoL. (Correlation coefficient $r = 0.75$, $P < 0.001$).

A AMML (I) peripheral blood cells, two showing black cytoplasmic deposits of reduced NBT (arrows) ($\times 1400$).

Fig. 5 Nitroblue tetrazolium reduction
B AMML (II) conglomeration of leukaemic cells, five of them showing black formazan deposits ($\times 1400$).

The reaction was negative in the characteristic 'hairy' cells of a case of leukaemic reticuloendotheliosis.

Two patients with acute myeloblastic leukaemia were infected at the time of study and their NBT reaction (in the blast cells) was as low as in the other non-infected patients with the same type of leukaemia. One patient with acute monoblastic-monocytic leukaemia was infected and the NBT reaction (56% of positive monocytes) was the highest of that group and that of type II acute myelomonocytic leukaemia. After *in vitro* stimulation with *E. coli* endotoxin, the number of NBT-positive monocytes increased from an average of 17% (resting) to 25% (stimulated); the corresponding increase in the proportion of NBT-positive neutrophils was from an average of 8% (resting) to 22% (stimulated). No correlation was found in acute myelomonocytic or monoblastic-monocytic leukaemia between the proportion of NBT-positive monocytes and that of neutrophils. A statistically significant correlation was found in

acute and chronic monocytic leukaemia between the proportion of NBT-positive and lysozyme-positive leukaemic cells (correlation coefficient $r = 0.76$, $P < 0.001$) (Fig. 6). Incubation with potassium cyanide did not produce inhibition of the NBT reaction of the monocytes (or the neutrophils) of three patients with acute myelomonocytic leukaemia (II) studied in the resting state and after endotoxin stimulation.

A positive NBT reaction was observed in approximately 10% of leukaemic monocytes after their macrophage transformation *in vitro* in three- to seven-day-old cultures (Fig. 7). This was only found after adding *Candida* to promote phagocytosis or endotoxin and not in the unstimulated cultures.

Discussion

The subjective element in the cytomorphological diagnosis of the acute leukaemias may be reduced by

Fig. 6 Correlation between the percentages of lysozyme and NBT-positive cells in AML and CMoL (correlation coefficient $r = 0.76$; $P < 0.001$).

Fig. 7 A seven-day monocyte culture stimulated with *E. coli* endotoxin. A black formazan deposit (arrow) shows the NBT reduction in one of the macrophages ($\times 1500$).

widening the range of investigative methods, the results of which may be expressed quantitatively, or which depend on the objective demonstration of a property associated with a specific line of cell differentiation. It is possible to distinguish lymphoblastic from the acute myeloid types in the great majority of cases, although in a small minority of cases the blast cells are undifferentiated and unclassifiable (Hayhoe, Quaglino, and Doll, 1964). In acute myeloid leukaemia, classification depends on the demonstration of features indicating granulocytic or monocytic differentiation or both in varying degrees (Schmalzl and Braunsteiner, 1971). Both methods described in the present work proved of help in the further characterization of the disease, especially of the monocytic component.

Asamer, Schmalzl, and Braunsteiner (1971) devised an immunofluorescent method of identifying lysozyme within the cells, and we have adopted the cytochemical method of Syren and Raeste (1971). Lysozyme is especially associated with monocytic differentiation. Our results with the cytochemical method for lysozyme help to define two major groups of acute myeloid leukaemia (Table). One includes cases in which the cells lack lysozyme activity (acute myeloblastic) or have only a moderate proportion of positive cells (acute myelomonocytic, type I), whilst the other includes cases in which approximately two-thirds of the leukaemic cells are positive (acute myelomonocytic type II and acute monoblastic-monocytic). Similar observations in a small number of cases of acute myeloblastic and monoblastic-monocytic were made by Perillie *et al* (1968). The number of circulating positive cells correlates well with the serum lysozyme concentrations (Fig. 4). A correlation between serum concentration and cellular lysozyme content was also found by Asamer *et al* (1971). Ohta and Nagase

(1971) found a positive relationship between leucocyte lysozyme content and the percentage of mature monocytes. We also find the highest proportion of positive cells (90 to 100%) in the chronic monocytic cases in which mature monocytes predominate. In addition to acute myeloid leukaemia, the cytochemical method has proved useful in other leukaemic disorders. A positive reaction in the leukaemic phase of a reticulum-cell sarcoma demonstrated the monoblastic nature of its poorly differentiated blast cells. In contrast, the reaction was negative in the blood and bone marrow cells of one case of poorly differentiated lymphocytic lymphoma and two of leukaemic reticuloendotheliosis. The latter finding would suggest that the tumour cell in the latter is of a different type from that in monocytic leukaemia.

The combined assessment of lysozyme, myeloperoxidase, and non-specific esterase activity permits a better characterization of the leukaemic cells, and so reduces the proportion of unclassifiable cases. When the leukaemic stem cells differentiate in the myeloblastic-promyelocytic direction, myeloperoxidase can be demonstrated at an earlier stage than lysozyme. A simultaneous positive reaction with both methods indicates a more advanced stage of cellular differentiation (late promyelocyte or early myelocyte). Early monoblastic-promonocytic differentiation may be demonstrated by a positive lysozyme and esterase activity, the latter reaction being sensitive to inhibition by NaF (Schmalzl and Braunsteiner, 1968; Daniel, Flandrin, Lejeune, Liso, and Lortholary, 1971). The correlation of the latter two methods in our hands has been fairly good. The possibility exists, however, that leukaemic cells, as part of their unbalanced behaviour, may not synthesize the enzymes simultaneously and that, in some cases, either lysozyme or non-specific esterase will prove more useful to define monocytic differentiation.

The metabolic changes which occur in neutrophils and monocytes after phagocytosis are associated with the reduction of NBT *in vitro* within the phagocytic vacuole (Nathan *et al*, 1969). The neutrophil NBT-reduction test has been shown to be a useful indication of bacterial infection (Park *et al*, 1968) and this is true also for the neutrophils from patients with acute myeloid leukaemia (Goldman and Catovsky, 1972). Not enough attention has been paid, however, to the NBT reduction of normal monocytes; perhaps they have been overlooked because of their small number in normal blood. We have demonstrated a positive NBT reaction in approximately one-third of monocytes from normal subjects as well as in those of patients with acute myelomonocytic (II), monoblastic-monocytic, and chronic monocytic leukaemia; few positive cells were seen in

acute myelomonocytic (I) and myeloblastic leukaemia (Table). The reaction in the latter groups was not related to the presence of infection; the number of positive leukaemic cells may be increased, however, in the groups with monocytic differentiation (as seen in one case of acute monoblastic-monocytic leukaemia). Both normal and leukaemic monocytes respond *in vitro* to endotoxin stimulation by a 1.4-fold increase in the proportion of positive cells; this increase was 2.5-fold in the neutrophils. The NBT reduction in monocytes was not inhibited by cyanide, thus suggesting that the enzyme responsible for the reaction is, as in the neutrophils, a cyanide-resistant one. The good correlation found between the proportion of lysozyme and NBT-positive cells (Fig. 6) further suggests that the latter test can be used as an indication of monocytic differentiation in acute myeloid leukaemia. A positive reaction was still elicited in some leukaemic monocytes after their transformation to macrophages *in vitro* (Fig. 7).

The two cytochemical methods described here help to confirm the impression obtained by other methods that in the great majority of cases it is possible to demonstrate cell differentiation in two main directions: (a) myeloblastic-promyelocytic (in acute myeloblastic and myelomonocytic (I)) and (b) monoblastic-monocytic (in acute myelomonocytic (II) and monocytic leukaemia). This can also be demonstrated by measuring serum and urine lysozyme concentrations and the total vitamin B₁₂-binding capacity (Catovsky, Ikoku, Galton, Griffin, and Hoffbrand, 1972b). These two types of acute myeloid leukaemia, which are a reflection of the two lines of cell differentiation, are also characterized by different degrees of extramedullary involvement by the disease: there is a significantly higher incidence of gum hypertrophy and lymphadenopathy in cases of type II acute myelomonocytic and monoblastic-monocytic leukaemia (Catovsky *et al*, 1972b). In addition, we have found the myeloperoxidase and the alkaline phosphatase content of mature neutrophil populations useful in identifying defective neutrophils presumably arising from leukaemic precursors; these cells are more common in the acute myeloblastic-myelomonocytic (I) group (Catovsky *et al*, 1972a).

The clinical value of subdividing acute myeloid leukaemia into these two broad groups according to the predominance of either granulocytic or monocytic differentiation remains to be established. Preliminary results suggest that the response to multiple-drug chemotherapy is better in the former group (Catovsky *et al*, 1972b).

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