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Altered isozyme patterns of leucocyte alkaline phosphatase in disease states

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SUMMARY. Leucocyte alkaline phosphatase (LAP) is a granulocyte enzyme whose concentration varies in disease states. In order to determine whether the pattern of expression is altered in leukaemic granulocytes, we have analysed the LAP isozyme pattern of a series of normal subjects and patients with various haematological diseases.

Electrophoretic patterns of partially purified LAP samples were determined by polyacrylamide gel electrophoresis in the presence of Triton X-100. These patterns were reproducible on repeated samples from the same patient. Presence of the LAP_f and LAP_s isozymes were determined after staining with the dye Fast Blue BB. Granulocytes were obtained from 15 normal subjects. Thirteen of these samples had only the LAP_s isozyme. The other two had LAP_s, plus a small amount ($\leqslant 10\%$ of total) of LAP_f activity. Eight patients with stable phase chronic myelogenous leukaemia (CML) had only small amounts of the normal LAP_s isozyme and no evidence of LAP_f. Of 11 patients with CGL who clinically had blast crisis, 10 had both LAP_s and LAP_f. The eleventh patient who was Ph^1 negative had only LAP_s. Three of five patients with polycythaemia vera had only the LAP_s isozyme while two had both isozymes. Six patients with non-malignant leucocytosis had only LAP_s. We interpret this data to indicate that the increased levels of LAP activity in some CGL blast crisis patients are primarily related to synthesis of the LAP_f isozyme.

Isozyme determinations have proved clinically useful in several instances. Creatine phosphokinase isozyme patterns are important in identifying organ-specific enzyme sources (Roberts & Sobel, 1978). Previous electrophoretic studies of LAP (Trubowitz & Miller, 1966; Lyons *et al*, 1968; Bottomley *et al*, 1969; Klein *et al*, 1966; Robinson *et al*, 1966) have demonstrated several LAP isozymes. These studies compared the starch gel electrophoretic patterns of LAP isolated from normal and diseased granulocytes. No consistent differences

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were noted from study to study. Using polyacrylamide gel electrophoresis we have demonstrated the occurrence of two isozymes of leucocyte alkaline phosphatase (LAP) and now address the question of whether they may be useful in distinguishing malignant and non-malignant diseases.

LAP is present in moderate amounts in normal granulocytes. This enzyme is unique from other organ-specific alkaline phosphatases (Miller & Yang, 1983) and has been partially purified.

The level of LAP enzymatic activity (determined histochemically) varies with different clinical conditions (Wachstein, 1946; Valentine *et al*, 1952, 1954; Trubowitz *et al*, 1959; Valentine & Beck, 1951; Koler *et al*, 1958; Mitus *et al*, 1958; Okun & Tanaka, 1979; Pritchard, 1957; Wiltshaw & Maloney, 1955; Spiers *et al*, 1975; Hayhoe & Quaglino, 1980). The enzyme is elevated in pregnancy, leucocytosis and Downs syndrome. The enzyme is absent or decreased in the leukaemic cells of patients with stable phase chronic granulocytic leukaemia. When these patients undergo blastic transformation, however, their leucocyte alkaline phosphatase may remain depressed or become normal or elevated.

After observing a single electrophoretic band of LAP activity in samples from normal granulocytes, we were surprised to observe a second, smaller LAP molecule present in the blast cells of a CGL blast crisis patient with elevated LAP score.

In this paper we report data obtained from LAP isozyme analysis of a series of normal subjects and patients with haematological diseases. Our results indicate that LAP_f may be a marker for CGL blast crisis and that the increased level of LAP observed in some CGL blast crisis patients may represent specific induction of the LAP_f isozyme.

METHODS

Granulocyte preparation. 15–50 ml of blood (depending on the white blood cell count) was drawn into a heparinized syringe. Mononuclear cells were removed by Ficoll-Hypaque density gradient centrifugation and erythrocytes by dextran sedimentation. The cells were pelleted by centrifugation at 2000 rpm in a tabletop centrifuge. The pellets were freed of residual erythrocytes by twice undergoing hypotonic lysis. This was done by resuspending the pellet in distilled water at 4°C for 15 s. The cell suspension was then adjusted to 0·14 M NaCl by the addition of 4 M NaCl.

Cord blood granulocytes were prepared in the same manner. 15 ml of heparinized umbilical cord blood was utilized in each case.

Partial enzyme purification. The white cell pellets were resuspended in 0·34 M sucrose and lysed by sonication for three 15 s bursts, each separated by 30 s. The LAP sample was partially purified by DEAE cellulose ion exchange chromatography prior to Triton–polyacrylamide gel electrophoresis. This step reduced the total protein applied to the gel, improving resolution, but did not alter the relative proportions of each band. In some cases, the sonicate was also applied directly to the Triton–polyacrylamide gel in order to ensure that altered electrophoretic patterns did not arise during the ion-exchange chromatography.

For DEAE-cellulose ion exchange chromatography, the sonicate was diluted 1:1 with 20 mM Tris, pH 9·4, 0·4% Triton X-100, 6 mM $MgCl_2$. The diluted sonicate was then applied to a

DEAE-cellulose column (3 × 10 cm) which had been equilibrated with starting buffer (10 mM Tris, pH 9·4, 0·2% Triton X-100, 3 mM $MgCl_2$). After sample application, the column was washed with starting buffer until no further protein was eluted. No LAP activity was eluted by this step. LAP was then eluted by washing with 0·15 N NaCl in starting buffer. 2 ml fractions were collected and LAP activity of each fraction measured. Peak fractions were pooled and precipitated with 2 volumes of acetone at −20°C overnight. Following centrifugation, samples were dissolved in electrophoresis buffer in preparation for gel electrophoresis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of Triton X-100 was performed by a modification of the method of Fishman (1974). The gels were 7% polyacrylamide with a running buffer of 0·1 M Tris–borate pH 9·5. Both the gel and running buffer contained 0·5% Triton X-100. The samples were applied in running buffer with 20% glycerol and run for 4 h at 175 V. Human placental alkaline phosphatase (PAP) (Sigma) was used as a standard in each gel. The gels were stained by incubation for 30 min at 37°C in 8·8 mM α-naphthyl acid phosphate in running buffer. Following this incubation, the gel was incubated at 37°C with 100 mg/ml Fast Blue BB. Bands were generally visible within 5 min.

Alkaline phosphatase assay. Alkaline phosphatase activity was assayed by measuring the release of p-nitrophenol from p-nitrophenylphosphate (Sigma) at 415 nm at 37°C, pH 9·5. The assay was performed in 0·5 M 2-amino-2-methyl-1-propanol, pH 9·5, 3 mM $MgCl_2$ and 5 mM p-nitrophenylphosphate. A unit of activity is defined as the release of 1 μmol of p-nitrophenol per minute.

RESULTS

Partial purification of LAP

The LAP samples which we obtained from a series of normal subjects and patients were partially purified by stepwise elution from a DEAE-cellulose ion exchange column. Fig 1

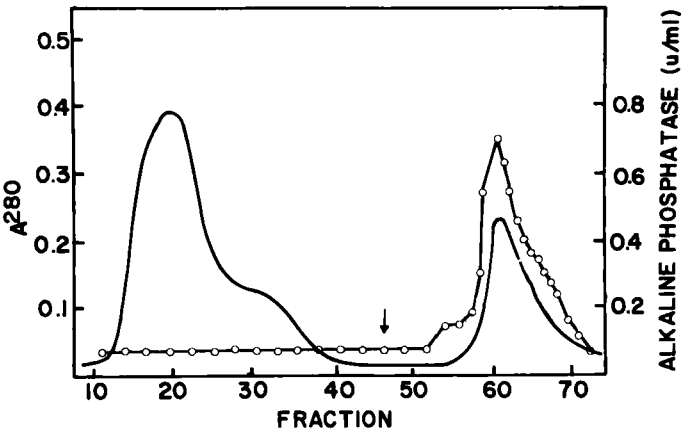

Fig 1. Partial purification of LAP by DEAE-cellulose ion exchange chromatography. Granulocyte sonicates were applied to a DEAE-cellulose column (3 × 10 cm) in starting buffer. LAP was eluted by the application of 0·15 N NaCl in starting buffer (arrow). —, A^{280}; O, LAP activity.

shows a typical elution pattern for this column. We typically observe a 5–10-fold purification by this procedure. In this case the specific activity was increased from 1·2 u/mg protein to 14·6 u/mg protein. The LAP activity containing fractions from this column were pooled and analysed by electrophoresis. This sample was from a patient with CGL blast crisis whose LAP demonstrated two isozyme bands by gel electrophoresis. The single peak demonstrates coelution of both isozymes from DEAE cellulose. This has been noted in each sample containing both isozymes.

Electrophoretic analysis of LAP isozymes

We have utilized an electrophoresis gel system which is a modification of that described by Fishman (1974). This system contains 0·5% Triton X-100 in both the gel and the running buffer. A typical gel is shown in Fig 2. Placental alkaline phosphatase (obtained commercially) consistently demonstrated two major bands of activity, in agreement with other studies of PAP isozymes (Mulivor *et al*, 1978). On some gels it was possible to see three sub-bands of enzymatic activity in the rapidly moving band. Normal LAP consistently demonstrated a single band which migrated only slightly faster than the slowly migrating

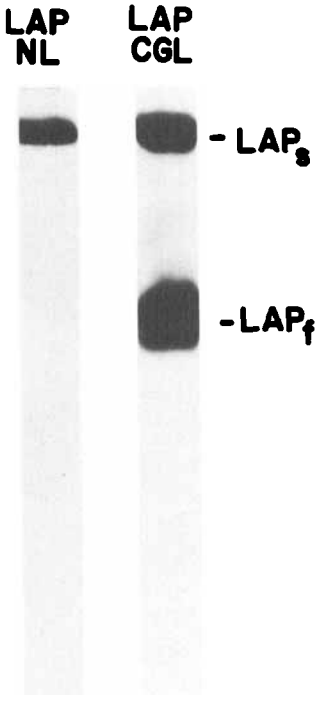

Fig 2. Triton–polyacrylamide gel electrophoresis of partially purified LAP from normal granulocytes and CGL cells. Triton–polyacrylamide gel electrophoresis and staining was performed as described in Methods.

band of PAP. The CGL sample, however, demonstrates two bands, the most intense of which is the rapidly migrating band. We have identified these two bands as LAP_s (slowly migrating band) and LAP_f (faster migrating band). Densitometry confirms that the majority of activity is in the LAP_f band.

LAP isozyme pattern of normal subjects

We have examined the LAP isozyme pattern of 15 normal subjects. The data presented in Table I demonstrates the presence of the slowly migrating isozyme in all of the samples. Two of the samples demonstrated a very slight activity of LAP_f ($\leqslant 10\%$ of normal activity). None of the subjects had a leucocytosis at the time of study.

Three cord blood granulocyte samples were also examined to determine whether LAP_f might represent a fetal isozyme of LAP. Each of the three samples demonstrated only LAP_s, despite elevated levels of enzyme activity.

LAP isozyme pattern of patients with leucocytosis

We have also determined the LAP isozyme pattern in the granulocytes of six patients with leucocytosis (Table II). Two of these patients had chronic infections and chronic granulocy-

Table I. LAP isozyme pattern of normal subjects and normal cord blood

Subject	LAP_s	LAP_f
1	+	−
2	+	±
3	+	−
4	+	−
5	+	−
6	+	−
7	+	−
8	+	−
9	+	−
10	+	−
11	+	−
12	+	±
13	+	−
14	+	−
15	+	−
Cord blood		
1	+	−
2	+	−
3	+	−

Table II. LAP isozyme pattern of patients with chronic leucocytosis

Patient	Diagnosis	LAP_s	LAP_f
1	Chronic infection	+	−
2	Chronic infection	+	−
3	Tumour, leucocytosis	+	−
4	Tumour, leucocytosis	+	−
5	Tumour, leucocytosis	+	−
6	Tumour, leucocytosis	+	−

tosis while four had tumour associated leucocytosis. All five of these patients demonstrated a single band of LAP activity, LAP_s.

LAP isozyme pattern of patients with CGL

Table III demonstrates the isozyme data which we have obtained from patients with CGL. We have separated the patients into two groups: those who were clinically in stable phase and those who were clinically in blast or accelerated phase. We have determined the isozyme pattern of eight patients with stable phase CGL, all of whom have only a single isozyme, LAP_s. We have also examined the pattern of 11 patients with the clinical diagnosis of blast phase CGL. Of these patients, 10 demonstrated the presence of two bands. LAP_f was present in relative concentrations of 15–70% of total enzyme activity. The patient with blast phase CGL who did not demonstrate the presence of LAP_f was a patient with inherited dwarfism and Ph^1

Table III. LAP isozyme pattern of patients with CGL

Stable phase CGL			Blast phase CGL			
Patient	LAP_s	LAP_f	Patient	LAP_s	LAP_f	Ph^1
1	+	−	1	+	+	+
2	+	−	2	+	+	ND
3	+	−	3	+	+	+
4	+	−	4	+	+	ND
5	+	−	5	+	+	+
6	+	−	6	+	−	−
7	+	−	7	+	+	ND
8	+	−	8	+	+	ND
			9	+	+	ND
			10	+	+	ND
			11	+	+	+

negative CGL. Patients 1, 3 and 6 had karyotypes performed and each of these patients had the Philadelphia chromosome.

We have also determined the LAP isozyme pattern of a small group of patients with other types of leukaemia (data not shown). Four patients with newly diagnosed acute myelogenous leukaemia had only LAP_s. Three patients with chronic lymphocytic leukaemia and one patient with acute lymphocytic leukaemia did not have alkaline phosphatase activity in their leukaemic cells. This suggests that the LAP_f isozyme may be specific for CGL and is not present in lymphocytic leukaemia cells.

LAP isozyme pattern of patients with polycythaemia vera (PCV)

We have determined the LAP isozyme pattern of five patients with PCV. The diagnosis of these patients had been confirmed by standard criteria. The results of their isozyme analysis are shown in Table IV. Two of the five patients had both LAP_f and LAP_s while the other three had only LAP_s.

Table IV. LAP isozyme pattern of patients with polycythaemia vera

Patient	LAP_s	LAP_f
1	+	−
2	+	+
3	+	+
4	+	−
5	+	−

DISCUSSION

It has been well documented that the normal level of LAP expression is altered in pathological conditions, being decreased in stable phase CGL and normal or increased in blast phase CGL. In this study we have demonstrated electrophoretic heterogeneity of LAP from normal subjects and patients with CGL and PCV. Our results suggest that the decreased level of LAP in stable phase CGL cells is related to decreased production of the isozyme normally present in granulocytes. The return to normal or elevated LAP activity in certain patients with blast crisis CGL seems to be related to the presence of LAP_f, those patients having elevated LAP scores exhibiting a predominance of LAP_f. The presence of LAP_f in blast crisis cells may account for the histochemical staining of LAP in blasts in some patients with CGL blast crisis as opposed to normal bone marrow is which they do not. Although the number of patients which we have studied is obviously too small to draw firm conclusions it appears that the altered LAP electrophoretic pattern may provide a marker for blast phase CGL cells.

CGL is a heterogeneous disease with $\geqslant 85\%$ of patients demonstrating the Ph^1 chromosome (Dallegri *et al*, 1979). Blast crisis CGL is even more heterogeneous with 10–15% of patients undergoing lymphoblastoid transformation at the initiation of blast crisis. It is possible that LAP_f is expressed only in Philadelphia chromosome positive CGL cells, since the single patient whom we studied with CGL blast phase who did not express LAP_f was the Ph^1 negative patient. Obviously the LAP isozyme pattern of many more patients will need to be examined to determine whether this is, in fact, the case.

It is clear that those patients having elevated LAP associated with leucocytosis do so on the basis of elevation of the normal isozyme while those patients with PCV may or may not express LAP_f.

Previous reports of electrophoretic heterogeneity of LAP (Robinson *et al*, 1966; Lyons *et al*, 1968; Klein *et al*, 1966) discussed the results of starch gel electrophoresis and in one case polyacrylamide gel electrophoresis (Bottomley *et al*, 1969) of LAP samples. These studies suggested enzyme heterogeneity but did not demonstrate a consistent pattern with specific disease states. The addition of Triton X-100 to both the polyacrylamide gel and the running buffer has enabled us to obtain reproducible gel patterns for LAP samples. Particularly important is the fact that all of the LAP activity migrates from the origin in this system.

The aetiology of the altered electrophoretic pattern of the partially purified LAP samples is unclear. Other organ-specific alkaline phosphatases demonstrate genetically determined isozymes which are functionally similar but electrophoretically and immunologically dissimilar (Gogolin *et al*, 1981). This appears to be the case with LAP as well.

Altered LAP gene expression in stable phase CGL cells is a rare example of a 'negative tumour marker', a normally synthesized protein which is present in the malignant cells in abnormally low amounts. Although the speculation concerning an extracellular factor repressing LAP synthesis (Rustin *et al*, 1980) remains unproven, there is clearcut evidence that these cells are capable of expressing normal or increased amounts of LAP. If these cells are maintained in tissue culture (Rustin *et al*, 1980; Dallegri *et al*, 1979; Kamada *et al*, 1981; Sato *et al*, 1982), if they are transfused into leucopenic patients (Levin *et al*, 1963), or if they are maintained in an intraperitoneal diffusion chamber (Chikkappa *et al*, 1973) they demonstrate increased levels of LAP after several days. The regulation of expression of these genes is clearly related to the clinical expression of the CGL phenotype.

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