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Flow cytometry of leucocyte alkaline phosphatase in normal and pathologic leucocytes

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Summary. Leucocyte alkaline phosphatase (LAP) is an enzyme expressed on the external aspect of the neutrophilic granulocyte plasma membrane, and represents a specific marker for the fully differentiated granulocyte. In this report we characterize 1B12.1, a monoclonal antibody raised against human bone alkaline phosphatase, by its ability to recognize the LAP protein. As assessed by Western blot analysis, following electrophoresis under non-reducing conditions, the antibody specifically reacts with LAP upon forced expression of the protein in simian COS-7 fibroblasts. In addition, the 1B12.1 antibody recognizes partially purified LAP isolated from peripheral blood granulocytes. With this antibody we developed a quantitative flow-cytometry-based method for the determination of LAP. Double fluorescence flow cytometry demonstrated that the LAP protein was present in relatively high amounts in neutrophilic granulocytes, but not in monocytes, natural killer cells, or B and T lymphocytes of normal individuals. The protein was completely absent in granulocytes obtained from chronic myeloid leukaemia and paroxysmal nocturnal haemoglobinuria patients. Higher than normal levels of LAP protein were evident in neutrophilic granulocytes of patients suffering from polycythaemia vera, essential thrombocythaemia and severe aplastic anaemia. However, the highest amounts of LAP protein were present in the granulocytes of normal individuals treated with G-CSF for the isolation of peripheral blood stem cells.

Keywords: alkaline phosphatase, diagnosis, flow cytometry, leucocyte, retinoic acid.

Leucocyte alkaline phosphatase (LAP) is a plasma membrane protein which belongs to the small family of alkaline phosphatase isoenzymes and is the product of the gene coding for the liver/bone/kidney-type (L/B/K-type) isoform (McComb *et al*, 1979; Weiss *et al*, 1988; Gianni' *et al*, 1994). LAP seems to be a specific marker for the post-mitotic neutrophilic granulocyte (Pedersen, 1982). The mRNA coding for the enzyme is absent in the monocytic, B- and T-lymphocytic lineages (Rambaldi *et al*, 1990). In addition, leukaemic cells representing different stages along the maturation process of neutrophilic granulocytes do not express significant amounts of LAP enzymatic activity and the corresponding mRNA (Rambaldi *et al*, 1990).

The amounts of LAP enzymatic activity are tightly regulated at various levels by different kinds of molecules (Gianni' *et al*, 1994, 1995a, b; Garattini & Gianni', 1996). In neutrophils, chemotactic agents rapidly export LAP to the plasma membrane from peculiar intracellular storage granules, where the protein is present in a catalytically inactive form (Borregaard *et al*, 1990, 1994). Granulocyte-colony-stimulating factor (G-CSF) causes the accumulation of LAP mRNA both in normal and in chronic myelogenous leukaemia (CML) granulocytes through a post-transcriptional mechanism (Rambaldi *et al*, 1990). In acute promyelocytic leukaemia (APL) cells, combinations of all-*trans* retinoic acid (ATRA) and G-CSF or cAMP stable analogues induce the expression of LAP by both transcriptional and early post-transcriptional effects (Gianni' *et al*, 1994, 1995a, b; Garattini *et al*, 1996).

Expression of LAP enzymatic activity in the neutrophilic granulocyte is known to be altered in various physiopathological conditions. High levels of the enzyme have been observed in the neutrophilic granulocyte during infection, pregnancy, and in various pre-leukaemic conditions, such

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as Fanconi anaemia and polycythaemia vera (PV) (McComb *et al*, 1979). In contrast, during the stable phase of CML and paroxysmal nocturnal haemoglobinuria (PNH), a specific defect in the expression of neutrophilic LAP, was observed (Rambaldi *et al*, 1989). Determination of LAP in peripheral blood neutrophils is diagnostically useful and it is a routine clinical laboratory procedure, which is performed with a semiquantitative assay known as the LAP score (Beutler, 1995; Hayhoe & Quaglino, 1958). A rapid and quantitative assay for the determination of LAP at the single-cell level in whole blood samples would be useful. In this report we describe the characterization of a mouse monoclonal antibody raised against human bone alkaline phosphatase (1B12.1 MoAb), which specifically recognizes the native and membrane-bound form of the LAP protein. With the use of this MoAb, we set up a flow-cytometry-based method for the quantitative determination of LAP in peripheral blood neutrophilic granulocytes. The method was used for the determination of LAP expression in normal and pathological blood samples.

MATERIALS AND METHODS

Patients. Whole blood samples were obtained from patients and healthy volunteers after informed consent. Four cases of PNH, six cases of CML, eight cases of essential thrombocythaemia (ET), eight cases of PV, and four cases of severe aplastic anaemia (AA) were analysed. Diagnosis of each disease was made according to standard criteria (The Italian Cooperative Study Group on Chronic Myeloid Leukemia, 1994; Cortelazzo *et al*, 1995a; Barbui *et al*, 1995; Camitta *et al*, 1982). In addition, samples from individuals treated with G-CSF for the mobilization of allogeneic (Rambaldi *et al*, 1996) or autologous (Cortelazzo *et al*, 1995b) peripheral blood progenitor cells (PBPC) were used. Informed consent was obtained from the patients and donors using forms approved by the Institutional Review Board. A full and detailed explanation of the potential risks and benefits concerning the collection of G-CSF mobilized PBPC for autologous and allogeneic transplantation was given to the donors and patients. Normal donors were >18 years old.

Reagents. Recombinant human G-CSF (specific activity 10^8 units/mg protein) was from Amgen Inc. (Thousand Oaks, Calif.). The 1B12.1 MoAb was obtained by immunization of mice with a highly purified preparation of human bone alkaline phosphatase as described elsewhere (Masuhara *et al*, 1992).

Transfection experiments. Simian COS-7 fibroblasts were obtained from American Type Culture Collection (ATCC, Rockville, Md.) and routinely passaged in Dulbecco-modified Eagle's medium (DMEM) containing 10% FCS. Transient transfection experiments were performed according to a standard calcium–phosphate coprecipitation procedure (Graham & Van der Eb, 1973) using the following two plasmids: pSG5 (Pharmacia, Uppsala, Sweden) and pSG5-LAP. The plasmid pSG5-LAP was constructed by inserting the full-length cDNA plasmid coding for the L/B/K-type alkaline phosphatase (ATCC; Weiss *et al*, 1986) into the EcoRI site of the pSG5 vector in the sense orientation relative to the SV40 promoter. Orientation of the insert was verified by cleavage with appropriate restriction endonucleases and by sequence analysis. 2 d after transfection of the plasmids, cells were harvested, lysed in gel-electrophoresis loading buffer (Laemmli, 1970) under non-reducing conditions and an aliquot of each extract was subjected to Western blot analysis using the 1B12.1 MoAb.

Partial purification of LAP from G-CSF mobilized peripheral blood leucocytes. Leucocytes (1×10^9 cells), obtained from the leukapheresis of a patient receiving G-CSF for the mobilization of autologous peripheral blood stem cells collection, were homogenized in 20 ml of 100 mM Tris-HCl (pH 7·6) containing 100 mM NaCl, 1 mM $MgCl_2$, 0·02 mM $ZnSO_4$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ by sonication. N-butanol, to a final concentration of 25% (v/v), was slowly added to the homogenate and the emulsion stirred at 4°C overnight. The mixture was centrifuged at 9000 ***g*** for 30 min and the aqueous layer containing LAP activity was separated from the organic phase. Cold acetone was added to the aqueous solution to a final concentration of 50%. The precipitate was sedimented by centrifugation, the pellet was dissolved in 5 ml of 50 mM Tris-HCl (pH) 7·0 and the clear solution was subjected to anion exchange chromatography on an FPLC MonoQ column (Pharmacia), equilibrated in the same buffer.

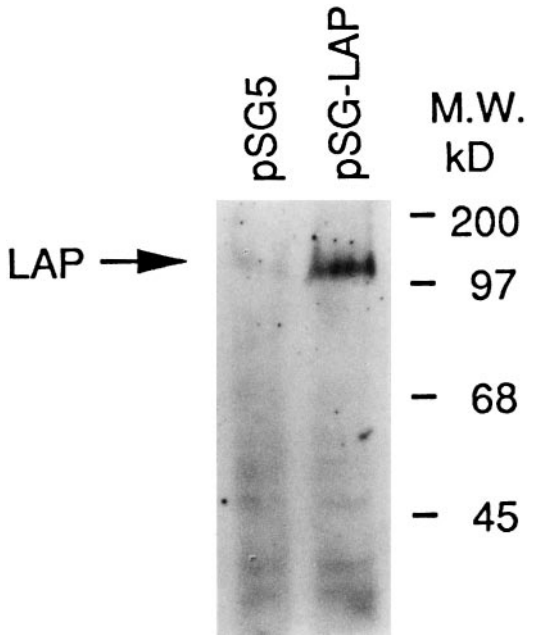

Fig 1. Western blot analysis of protein extracts derived from COS-7 cells transfected with LAP cDNA. COS-7 cells were transiently transfected with either pSG5 or PSG5-LAP using the calcium phosphate co-precipitation method. 48 h after transfection, total cellular extracts were prepared and subjected to Western blot analysis under non-reducing conditions using the 1B12.1 MoAb. The position of the protein band recognized by the 1B12.1 MoAb is indicated by the arrow on the left. The position of the molecular weight marker proteins is indicated on the right (myosin, 200 kD; phosphorylase B, 97 kD; bovine serum albumin, 68 kD; ovalbumin, 45 kD).

Chromatography was performed at a flow rate of 1 ml/min and LAP activity was eluted at about 350 mM NaCl in 12 fractions of 1 ml each. An aliquot of each fraction was subjected to LAP enzymatic activity determination and Western blot analysis with the 1B12.1 MoAb. Following chromatography on the MonoQ column, approximately 50% of the LAP enzymatic activity present in the original homogenate was recovered.

LAP flow cytometry and LAP score assays. Immunofluorescence analysis was performed on 50 μl of heparinized whole blood incubated for 30 min at 4°C with the following antibodies: 1B12.1 (IgG1), OKM1 (IgG2b, anti-CD11b, obtained from the ATCC), 1D3 (Ord *et al*, 1990) (IgG2b, anti-CD16, obtained from J. D. Griffin, Dana Farber Cancer Institute, Boston, Mass.) and isotypic matched negative controls (Becton Dickinson, Mountain View, Calif.). 50 μl samples of whole blood were incubated for 30 min at 4°C with the primary antibody. After washing with medium containing 2·5% human AB serum, cells were pelleted and resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Becton Dickinson, Mountain View, Calif.). The cells were further incubated for 30 min at 4°C and the erythrocytes were lysed with NH_4Cl buffer (NH_4Cl 8·99 g/l, $KHCO_3$ 1 g/l, Na_4EDTA 0·037 g/l, pH 7·3) for 5 min at room temperature. The cells were then washed twice in phosphate-buffered saline (PBS) and then analysed by flow cytometry using a FACScan analyser (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm, power emission of 150 mW, and the filter set for FITC colour fluorescence. Experiments with double-fluorescence staining were performed as above with a further incubation for 30 min at 4°C, using the following lineage-specific phycoerythrin-conjugated monoclonal antibodies: Leu15 (anti-CD11b) for granulocytes, Leu4 (anti-CD3) for mature T cells, Leu12 (anti-CD19) for B cells, LeuM3 (anti-CD14) for monocytes, Leu11c (anti-CD16) for NK cells. All these monoclonal antibodies were from Becton Dickinson. FACS analysis on granulocytes, lymphocytes and monocytes was performed by specific gating of the cells on the basis of side and forward light scatter.

LAP scores were cytochemically determined according to Kaplow as modified by Hayhoe & Quaglino (1958).

Measurement of LAP activity. Cells were harvested, pelleted by centrifugation at 400 ***g*** for 10 min, washed once with 0·9% NaCl and centrifuged again. The washed cell pellet was

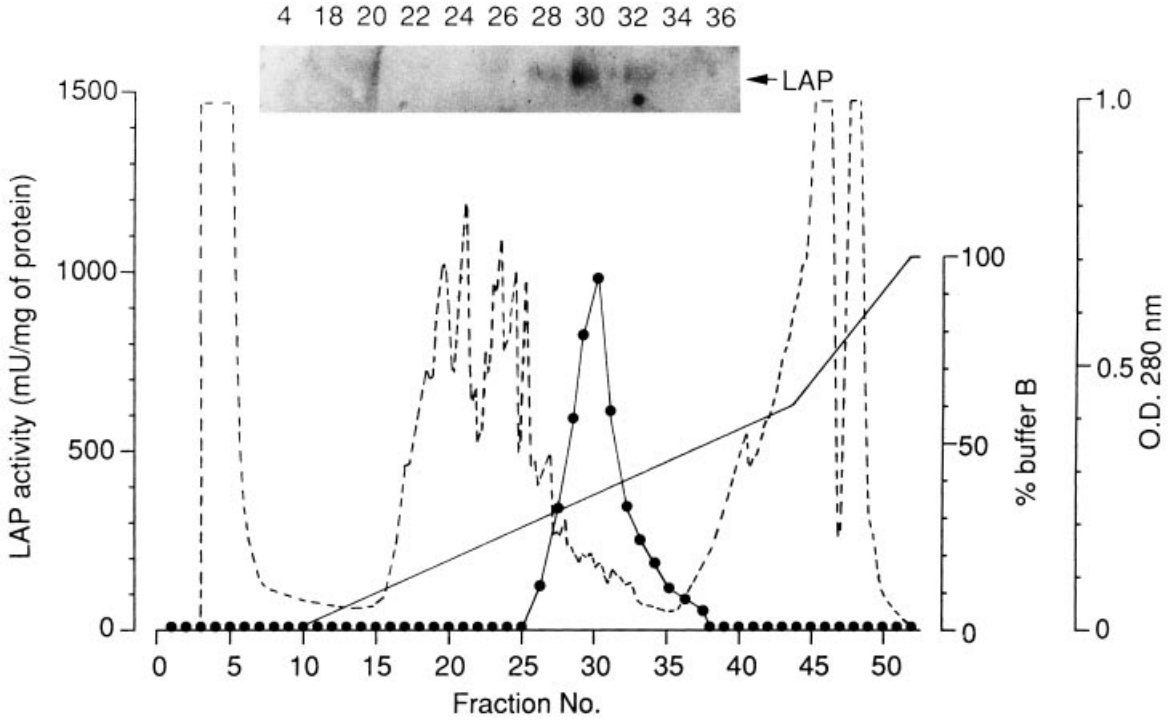

Fig 2. Chromatographic separation of LAP enzymatic activity and determination of the specificity of the 1B12.1 MoAb. Peripheral blood leucocyte extracts were loaded on a MonoQ anion exchange FPLC column equilibrated with 50 mM Tris/HCl (pH 7·0). LAP enzymatic activity was eluted with a gradient of NaCl (0–1·0 M; buffer B, solid line) superimposed on the equilibration buffer. The total protein elution profile was monitored at 280 nm with an on-line UV spectrophotometer (dashed line chromatogram). 1 ml fractions were collected and an aliquot of each fraction was used for the determination of LAP activity (solid dots and solid line chromatogram). One milliunit (mU) of LAP enzymatic activity is defined as the amount of enzyme capable of transforming 1 nmole of substrate in 1 min at 25°C. An aliquot of the indicated fractions was subjected to Western blot analysis under non-reducing conditions, using the 1B12.1 MoAb. Immunoreactive bands were revealed by use of a chemiluminescent substrate after incubation with a secondary antibody linked to horseradish peroxidase. Although all the fractions of the chromatogram were subjected to Western blot analysis, only selected ones are indicated in the inset figure. The position of the 1B12.1 immunoreactive LAP band is indicated by the arrow on the right and corresponds to a molecular weight of approximately 150 kD.

resuspended in homogenization buffer (1 mM $MgCl_2$/1 mM $CaCl_2$/20 mM $ZnCl_2$/0·1 M NaCl/0·1% (v/v) Triton X-100/50 mM Tris/HCl, pH 7·4) and disrupted by vigorous pipetting. The homogenate was used for the LAP assay, which was performed in alkaline conditions (pH 9·8) with p-nitrophenol phosphate (Sigma) as substrate according to the manufacturers' instructions. LAP activity was normalized for the content of protein in the sample. Proteins were measured according to the Bradford method (Bradford, 1976) using BSA fraction V (Sigma) as a standard. One unit of LAP activity is defined as the amount of enzyme capable of transforming 1 nmole of substrate in 1 min at 25°C. Enzyme assays were performed in conditions of linearity relative to the substrate and to the concentration of proteins.

Western blot analysis. Aliquots of COS-7 cell homogenates and MonoQ chromatographic fractions were resuspended in loading buffer without boiling and reduction with 2-mercaptoethanol, and electrophoresed on an 8% SDS-denatured polyacrylamide gel. Proteins were electro-blotted on nitrocellulose membranes (Schleicher and Schuell, Darmstadt, Germany) according to standard protocols (Kurosaki *et al*, 1995). Immunoreactive protein bands were revealed by a chemiluminescence-based procedure using the ECL detection kit (Amersham, Little Chalfont, U.K.) according to the manufacturers' instructions.

RESULTS

Characterization of a monoclonal antibody recognizing LAP

The 1B12.1 MoAb was originally raised against human purified bone alkaline phosphatase (Masuhara *et al*, 1992). The gene coding for the L/B/K-type alkaline phosphatase is the same as that responsible for the expression of bone alkaline phosphatase in osteoblasts and LAP in human neutrophils (McComb *et al*, 1979; Gianni' *et al*, 1994). To confirm that the 1B12.1 MoAb is directed against the product of the L/B/K-type alkaline phosphatase gene, we performed transient transfection experiments in simian COS-7 fibroblasts with a construct (pSG5-LAP) containing the corresponding cDNA under the control of a strong constitutive promoter. COS-7 was chosen as the recipient cell line because it is known to contain only trace amounts of endogenous alkaline phosphatase activity. As shown in Fig 1, COS-7 cells transfected with the eukaryotic expression vector alone (pSG5) did not express significant amounts of 1B12.1 cross-reactive protein, as assessed by Western blot analysis in non-reducing conditions. In contrast, fibroblasts transfected

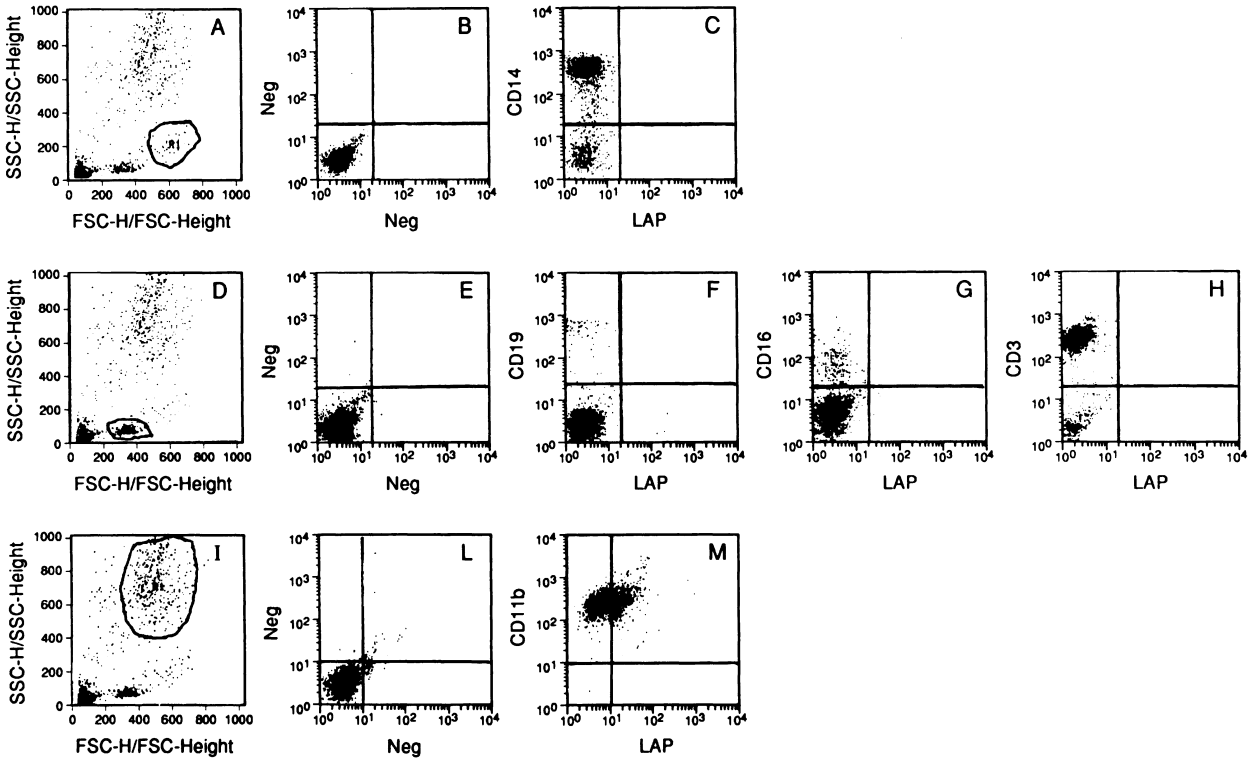

Fig 3. Double-fluorescence flow cytometry experiments on monocytic, granulocytic and lymphocytic peripheral blood cell populations. Peripheral blood cell populations were incubated with 1B12.1 MoAb (panels C, F, G, H and M) or with an isotype-matched irrelevant MoAb (panels B, E and L) and subsequently stained with a fluorescein-conjugated anti-mouse Ig. Thereafter, the same samples were treated with phycoerythrin-conjugated lineage-specific MoAb recognizing the indicated antigens (CD14 for the monocytes, panel C; CD19 for B lymphocytes, panel F; CD16 for NK cells, panel G; CD3 for T lymphocytes, panel H; CD11b for granulocytes, panel M), and with isotype-matched phycoerythrin or fluorescein-conjugated control antibodies (Neg, panels B, E and L). Monocytic (panel A), lymphocytic (panel D) and granulocytic (panel I) cell populations were selected by specific gating on the basis of the forward (FSC-H/FSC-Height) and side (SSC-H/SCC-Height) light scatter.

with the same vector containing the full-length cDNA coding for L/B/K-type alkaline phosphatase demonstrated the presence of a single 150 kD protein specifically recognized by the 1B12.1 MoAb. When the same experiment was conducted under reducing conditions, 1B12.1 MoAb was unable to recognize the protein (data not shown). Thus, our data indicated that the antibody was directed against the product of the L/B/K-type alkaline phosphatase gene, and the conformation of the specific epitope was crucial for its reactivity with the MoAb.

To further demonstrate that the 1B12.1 MoAb specifically recognizes LAP, the protein was partially purified from peripheral blood neutrophils of G-CSF-treated normal donors. As shown in Fig 2, LAP enzymatic activity was eluted as a single peak on an anion exchange column. Fractions containing LAP enzymatic activity show a 150 kD protein specifically recognized by the 1B12.1 MoAb, as assessed by Western blot analysis. The intensity of the cross-reacting bands coincided with the levels of LAP enzymatic activity present in each fraction (only peak fractions are presented in Fig 2). These data demonstrated that 1B12.1 MoAb recognized the alkaline phosphatase form expressed in normal human neutrophils.

Development of a flow cytometric assay for LAP

We developed a quantitative flow-cytometry-based method for the determination of LAP in whole blood samples. Measurement of LAP relies on an indirect labelling technique involving 1B12.1 used as the primary antibody and a fluorescein-conjugated anti-mouse Ig as the secondary antibody. Since LAP is believed to represent a specific marker of the terminally differentiated neutrophilic granulocytes (Pedersen, 1982; Rambaldi *et al*, 1990), experiments were performed to confirm this lineage specificity. As shown in Fig 3, double-fluorescence assays performed with 1B12.1 and lineage-specific monoclonal antibodies demonstrated the presence of significant amounts of LAP only on mature neutrophils. LAP in T lymphocytes, B lymphocytes, NK cells and monocytes was not detected with this monoclonal antibody.

As shown in Fig 4, flow cytometry of normal neutrophils demonstrated that these cells expressed significant amounts of LAP protein and CD11b, a pan-myeloid surface marker used as a control in this set of experiments. In contrast, granulocytes obtained from a PNH patient were characterized by a defective expression of LAP and CD16, which was accompanied by normal plasma membrane levels of CD11b. These data are in line with the fact that LAP (Selvaraj *et al*, 1988) and CD16 (Bessler *et al*, 1994) are PIG-tailed proteins and PNH is associated with a defect in the assembly and surface export of this type of proteins (Miura *et al*, 1994). Similarly, neutrophils obtained from the peripheral blood of patients with CML during the stable phase of the disease were devoid of LAP associated fluorescence. In contrast, other

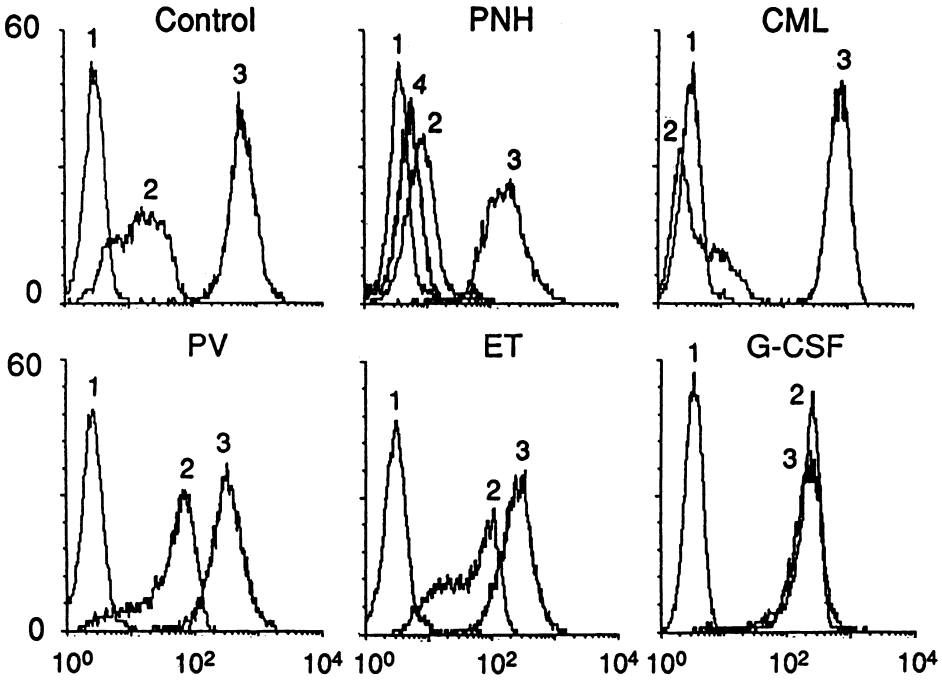

Fig 4. Determination of LAP-positive cells by flow cytometry with 1B12.1 MoAb in normal individuals and selected haematological patients. Representative flow-cytometry profiles of granulocytes obtained from a normal individual (Control), a G-CSF-treated normal donor (G-CSF), and patients suffering from essential thrombocythaemia (ET), paroxysmal nocturnal haemoglobinuria (PNH), chronic myeloid leukaemia (CML) and polycythaemia vera (PV). Tracing 1 = cells treated with an isotype-matched antibody (negative control); tracing 2 = cells treated with the 1B12.1 MoAb; tracing 3 = cells treated with a MoAb recognizing the myeloid surface marker CD11b; tracing 4 = cells treated with a MoAb recognizing the PIG granulocytic surface marker CD16.

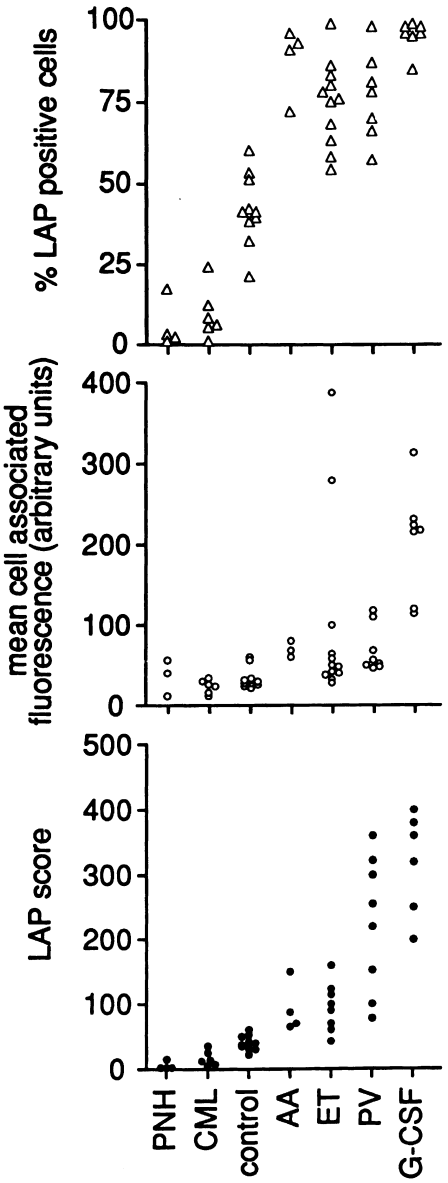

Fig 5. Determination of LAP by the 1B12.1 MoAb-based flow cytometry assay and by the LAP score method in peripheral blood preparations obtained from normal individuals and selected haematological patients. Peripheral blood preparations obtained from normal individuals (Control), from leukaemic patients treated with G-CSF for the mobilization of haemopoietic stem cells (G-CSF) and patients suffering from paroxysmal nocturnal haemoglobinuria (PNH), chronic myeloid leukaemia (CML), polycythaemia vera (PV), essential thrombocythaemia (ET) and aplastic anaemia (AA), were subjected to flow cytometric analysis of LAP using the 1B12.1 MoAb (upper two panels) or to the determination of LAP score (lower panel, solid circles). The flow cytometric analysis of LAP is expressed relative to the number of 1B12.1 MoAb-positive cells (open triangles) or relative to CAMF (open circles). Each symbol in the three graphs represents a separate individual.

Table I. Evaluation of LAP in human normal and pathologic neutrophilic granulocytes by histochemistry and flow cytometric analysis using the 1B12.1 MoAb.

	LAP score	1B12.1 positive cells (%)	1B12.1 CAMF (arbitrary units)
Control	38 (25–125)	41 (21–60)	30 (24–60)
G-CSF	360 (200–400)	96 (85–98)	218 (114–315)
PNH	4 (0–15)	3 (1–17)	40 (12–56)
CML	12 (5–35)	7 (1–12)	26 (17–30)
PV	256 (78–360)	81 (57–98)	56 (46–118)
ET	70 (42–124)	76 (58–80)	48 (29–389)
AA	79 (46–150)	92 (72–96)	64 (60–81)

Heparinized whole blood samples were obtained from the normal individuals and the patients shown in Fig 5 and subjected to the determination of LAP using the LAP score and flow cytometric methods. The flow cytometric analysis of LAP is expressed relative to the number of 1B12.1 MoAb-positive cells (1B12.1-positive cells) or relative to the cell associated mean fluorescence (1B12.1 CAMF). Results are the median value of each set of data with the intervals of confidence in parentheses. Control = normal individuals; G-CSF = leukaemic patients treated with G-CSF for the mobilization of haemopoietic stem cells; PNH = patients suffering from paroxysmal nocturnal haemoglobinuria (PNH); CML = chronic myeloid leukaemia; PV = polycythaemia vera; ET = essential thrombocythaemia; AA = aplastic anaemia; CAMF = cell-associated mean fluorescence.

types of chronic myeloproliferative disorders like PV and ET are usually associated with high levels of LAP gene expression and enzymatic activity. Concordantly, flow cytometric analysis of neutrophils obtained from these patients showed high levels of positivity for the 1B12.1 MoAb. Finally, stimulation of granulocytopoiesis by *in vivo* administration of G-CSF to mobilize PBPC resulted in the appearance of extremely high levels of 1B12.1-associated fluorescence.

To evaluate whether results obtained by quantitative flow cytometry of LAP protein paralleled those obtained by the conventional cytochemical LAP score method, a series of experiments were conducted on neutrophils obtained from normal volunteers, G-CSF-treated patients and patients suffering from CML, PNH and myeloproliferative disorders. The results of this analysis are presented in Fig 5 and summarized in Table I. The flow cytometric data are expressed relative to the number of LAP-positive cells and single cell mean associated fluorescence (CAMF). As demonstrated by Fig 5, there was a general concordance among the various test groups, between the results obtained by the LAP score method and the number of cytofluorometrically determined LAP-positive cells and CAMF. The parallel between LAP score and the two flow cytometry determined parameters was more evident when the median values of each group of individuals were analysed (Table I).

DISCUSSION

Determination of LAP activity is routinely used for the laboratory diagnosis of different haematological diseases

such as CML, PV, idiopathic myelofibrosis and leukaemoid reactions (Beutler, 1995). The enzymatic activity is determined in clinical laboratories by means of a qualitative cytochemical method known as the LAP score. The test is based on the LAP-catalysed hydrolysis of a phosphate derivative of aryl naphtholamide which is coupled to a diazonium salt, leading to the formation of an insoluble dye readily visualized over neutrophilic leucocytes that contain alkaline phosphatase activity. The test is performed on fixed blood films that are counterstained with haematoxylin. With the use of qualitative morphologic criteria, 100 neutrophilic leucocytes are scored from 0 to 4+ on the basis of the precipitated dye in their cytoplasm. The method is at best semi-quantitative, tedious, and subject to great inter-laboratory variability. The normal range for the LAP-score test needs to be determined in each laboratory and may vary with the operator, different coupling dyes and different dye lots. Introduction of a quantitative and reproducible assay that allows quantitative measurement of LAP would represent an important advance in the diagnostic haematological routine. In this report we characterized a monoclonal antibody, 1B12.1, originally developed against human bone alkaline phosphatase (Masuhara *et al*, 1992). We demonstrated that 1B12.1 MoAb specifically recognized both recombinant and natural LAP. In addition, we showed that the antibody can be used to quantitatively determine LAP by flow cytometric analysis of unfractionated heparinized peripheral blood. Upon Western blot analysis, 1B12.1 MoAb recognized LAP only following electrophoresis under non-reducing conditions, suggesting that the antigenic epitope is, at least partially, of conformational nature. It is also likely that our antibody recognized enzymatically active LAP, which is the only form of the protein present on the plasma membrane of neutrophils (Borregaard *et al*, 1990, 1994).

The quantitative flow cytometric assay for LAP was used to confirm, at the protein level, the neutrophil cell specificity of LAP expression using normal blood leucocyte subpopulations (Fig 3). In addition, with this method, we measured LAP protein in whole blood samples obtained from normal volunteers as well as patients suffering from preleukaemic and leukaemic diseases associated with lower and higher than normal levels of LAP enzymatic activity. As expected, neutrophilic granulocytes obtained from PNH (Rambaldi *et al*, 1989) and CML (Rambaldi *et al*, 1989, 1990) patients were characterized by a very small number of LAP-positive neutrophils with a limited amount of LAP expression on each individual cell. The data obtained in PNH granulocytes demonstrated that the defect in LAP enzymatic activity, typical of this disease, was due to a defective export of the corresponding protein to the plasma membrane and not to mutations affecting the catalytic activity of the polypeptide. This finding is in line with the fact that LAP is a PIG protein and PNH is characterized by a deficit in the assembly and the export of PIG proteins to the plasma membrane (Bessler *et al*, 1994). In contrast, the results obtained in CML granulocytes during the stable phase of the disease, were consistent with the lack of LAP mRNA observed in these leukaemic cells (Rambaldi *et al*, 1989, 1990). Nevertheless, in the CML setting, since the flow cytometry assay is performed on all granulocytes, a potential problem of high numbers of peripheral blood immature myeloid cells may lead to artificially low LAP values. This could be avoided by the development of a double-fluorescence method by which LAP and the neutrophil-specific marker CD16 could be simultaneously determined.

The high levels of LAP enzymatic activity observed in the granulocytes obtained from PV, ET and AA patients are the consequence of an increase in the levels of the corresponding protein and not due to activation of the enzyme. The molecular mechanism underlying the higher than normal levels of LAP observed in these three myeloproliferative disorders are not yet known. However, it is interesting to note that LAP is tightly controlled by G-CSF, a cytokine whose receptor is associated with the Jak2 tyrosine kinase, and the Stat3 transcription factor. These are the same elements of the signal transduction systems used by the erythropoietin receptor (Miura *et al*, 1994), whose intracellular signalling system is probably altered in some cases of PV (Sokol *et al*, 1995). In addition, it is worth mentioning that AA patients have been recently reported to have higher than normal levels of circulating G-CSF (Kojima *et al*, 1996).

By far the highest levels of LAP protein were present in the peripheral blood myeloid cells mobilized from the bone marrow upon *in vivo* treatment with G-CSF. In this condition, the peripheral blood is characterized by the appearance of a remarkable number of intensively stained LAP-positive cells. These LAP-positive cells have an associated level of LAP-dependent fluorescence which is between 4- and 10-fold higher than that observed in neutrophilic granulocytes obtained from normal individuals. The data strongly suggest that *in vivo* treatment with G-CSF results in the rapid maturation of granulocytic precursors in which synthesis or plasma membrane export of LAP is dramatically induced. Interestingly, double-fluorescence experiments have demonstrated that LAP-positive G-CSF mobilized cells are also CD16 positive, which indicates co-regulation of the two markers of the terminally differentiated granulocyte (unpublished observations).

Having demonstrated the usefulness of LAP as a differentiation marker for the granulocytic maturation of leukaemic cells (Gianni' *et al*, 1994, 1995b; Rambaldi *et al*, 1990), the availability of a flow cytometric assay for the determination of this enzyme can be exploited to perform *in vivo* and *ex vivo* studies on the expression of the antigen following cyto-differentiating treatments of myeloid leukaemias. In addition, the simplicity and reproducibility as well as the quantitative nature of the flow cytometric assay for LAP is well suited for a routine haematological laboratory.

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