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Differential Suppression of Background Mammalian Lysosomal β-Galactosidase Increases the Detection Sensitivity of *LacZ*-Marked Leukemic Cells

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A method is described for the detection of *Escherichia coli* β-galactosidase-expressing leukemic cells in *ex vivo* bone marrow samples. 4-Methylumbelliferyl-β-D-galactopyranoside is used as a substrate in a kinetic assay. D-Galactose is used to suppress endogenous lysosomal β-galactosidase activity, yielding a sixfold increase in sensitivity. With this assay, the detection limit is one leukemic cell per 10^4 normal bone marrow cells.
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Although in recent years treatment protocols for acute myelocytic leukemia have been substantially improved, many patients still develop a leukemic relapse, caused by the regrowth of leukemic cells that survive the treatment. More knowledge of the localization and the growth pattern of these very low numbers of residual surviving cells may help in improving the treatment protocols.

The brown Norway rat acute myelocytic leukemia model (BNML)[1] and its *in vitro* growing subline LT12 have been extensively characterized and accepted as a relevant animal model for human acute myelocytic leukemia (1). Recently, a subline LT12NL15 was developed from the LT12 cell line, by using the BAG vector (2) to introduce the *Escherichia coli* β-galactosidase (*LacZ*) gene into its genome. LT12NL15 cells exhibit stable, high-level expression of *E. coli* β-galactosidase in every individual cell, *in vitro* as well as *in vivo* (3). These genetically marked cells are used to study the *in vivo* homing behavior and growth patterns of leukemia cells in the BNML model. In these studies, it is essential to have ways of detecting very low numbers of the genetically marked cells in tissue samples containing very large numbers of cells.

Most reports describing assays for the detection of *LacZ* expression in transfected cells have focused on the sensitive detection and sorting of single *LacZ*-expressing cells using flow cytometry (4,5) or on the detection of β-galactosidase in lysates (6,7). The detection of *LacZ*-marked cells *in vivo* is so far almost exclusively based on the histochemical detection of β-galactosidase expression using X-gal as a substrate (8). These assays rely on visual inspection of the samples, which is rather time consuming and limits their applicability.

We report here the development of a kinetic β-galactosidase assay using 4-methylumbelliferyl-β-D-*galacto*pyranoside (MUG) as a substrate, with which bone marrow samples can be assayed for the presence of *LacZ*-marked leukemic cells. When detecting *E. coli* β-galactosidase activity in mammalian tissue samples, the detection sensitivity is limited by the presence of considerable amounts of endogenous lysosomal β-galactosidase. Several authors, each describing the characterization of a specific β-galactosidase enzyme, have reported compounds that suppress the respective enzyme (9). We have compared a number of these compounds for their ability to selectively suppress rat lysosomal β-galactosidase vs *E. coli* β-galactosidase. None of these compounds showed such an effect. However, we did find that product inhibition by D-galactose can cause a sixfold differential suppression of lysosomal β-galactosidase vs *E. coli* β-galactosidase. Using our optimized assay we can detect 400 LT12NL15 cells in the presence of 5×10^6 normal bone

[1] Abbreviations used: BNML, brown Norway rat acute myelocytic leukemia model; *LacZ*, *E. coli* β-galactosidase gene; BM, bone marrow; PBS, phosphate-buffered saline; CBS, citrate-buffered saline; Tris, tris-hydroxymethylaminomethane; MUG, 4-methylumbelliferyl-β-D-galactopyranoside; FIR, fluorescence intensity increase rate; 4-MU, 4 methylumbelliferon; X-gal, 5-bromo-4-chloro-3-phenylindolyl-β-D-galactopyranoside; AMPGD, 3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo [3.3.1.1^{3,7}]decan-4-yl)phenyl-β-D-galactopyranoside; DMSO, dimethyl sulfoxide.

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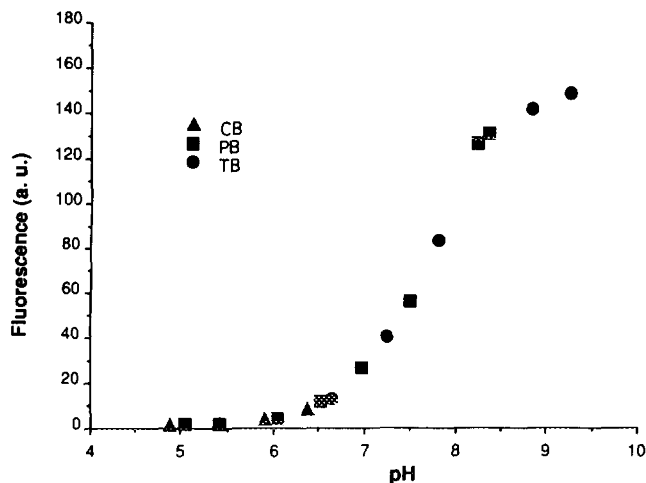

FIG. 1. Effect of pH on fluorescence efficiency of 10 ng/ml 4-methylumbelliferon. CB, citrate-buffered saline; PB, phosphate-buffered saline; TB, Tris-buffered saline.

marrow cells, the standard sample size used in the assay. This implies a lower detection limit of one leukemic cell per 10^4 normal cells.

MATERIALS AND METHODS

Brown Norway rats from the BN/BiRij strain were bred at the Medical Biological Laboratory TNO (Rijswijk, The Netherlands). LT12 and LT12NL15 cells were cultured in α-MEM supplemented with 10% fetal calf serum (Gibco, Breda, The Netherlands). LT12NL15 cells were used as a source of *E. coli* β-galactosidase. 4-Methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferon (4-MU) were purchased from Molecular Probes (Eugene, OR). D-Galactose, dithionitrobenzene, D-galactonic acid, D-galactal, and Triton X-100 were purchased from Sigma (St. Louis, MO). Fluorescence was measured with a Perkin–Elmer LS50 fluorometer (Perkin–Elmer, Beaconsfield, UK). Excitation was set to 365 nm, bandwidth 15 nm; emission was set to 440 nm, bandwidth 5 nm.

4-MU fluorescence vs pH. 4-MU was dissolved in DMSO at a concentration of 1 mg/ml. Of this solution 20 μl was mixed with 2 ml of citrate-buffered saline (CB; 100 mM sodium citrate, 0.9% NaCl), phosphate-buffered saline (PB; 100 mM sodium phosphate, 0.9% NaCl), or Tris-buffered saline (TB; 100 mM Tris–HCl, 0.9% NaCl) covering a pH range of 4.9 to 9.3. The fluorescence intensity of the 4-MU was measured as a function of the pH.

Bone marrow cells. Rats were killed using carbon dioxide and femora were taken out and kept on ice. Bone marrow suspensions were prepared by flushing the femora with ice-cold PBS (PBS; 12.7 mM sodium phosphate, 0.82% NaCl, pH 7.4). To remove red blood cells the samples were mixed with an equal volume of ice-cold erythrocyte lysis buffer and incubated for 10 min on ice. Subsequently, the cells were washed three times with PBS. Composition of the erythrocyte lysis buffer was 155 mM NH_4Cl, 11.9 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4. After removal of red blood cells bone marrow samples were washed in PBS.

Lysates. Lysates were prepared by resuspending the cells in 0.1% Triton X-100 in water, in a volume corresponding to 5×10^7 cells/ml. This was followed by vigorous stirring for 1 min. Finally, the lysates were centrifuged for 15 min at 1500*g* to remove particulate matter.

MUG assay. MUG was dissolved in DMSO at a concentration of 50 mM. Of this MUG solution 20 μl was pipetted into a quartz cuvette, and reaction buffer and cell lysate were added to a final volume of 2 ml. Reaction buffer was 100 mM Tris–HCl of varying pH, containing 2 mM $MgCl_2$ and variable concentrations of D-galactose. After thorough mixing, the increase of fluorescence intensity was recorded for 60 s. The fluorescence intensity increase rate (FIR) was calculated by linear regression analysis of the fluorescence intensity vs time curve and taken as a measure of β-galactosidase activity, expressed as arbitrary units (a.u.).

Inhibitors. Dithionitrobenzene, galactal, D-galactonic acid, and D-galactose were explored for their potential use as selective inhibitors of lysosomal β-galactosidase activity. Concentrations were used that were stated in the literature to be effective in suppressing the respective β-galactosidase (9). In addition, product inhibition by D-galactose was tested for its differential effect

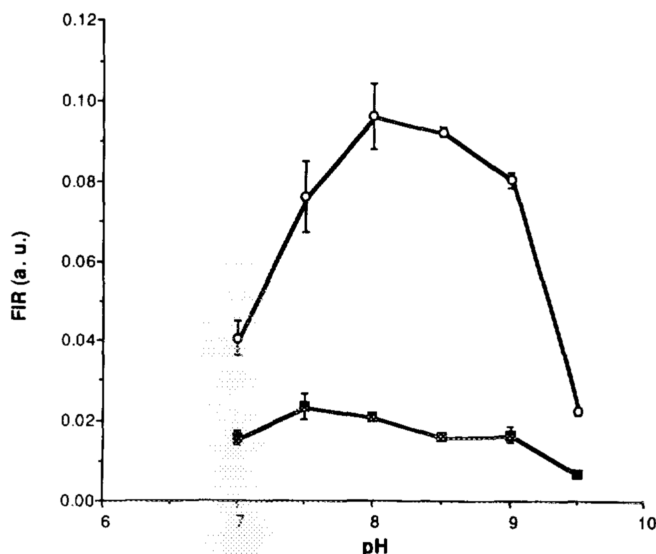

FIG. 2. Effect of pH on fluorescence increase rate (FIR) of lysates from 1×10^4 LT12NL15 cells, with and without the addition of 100 mM D-galactose. Error bars represent standard deviations of triplicate measurements.

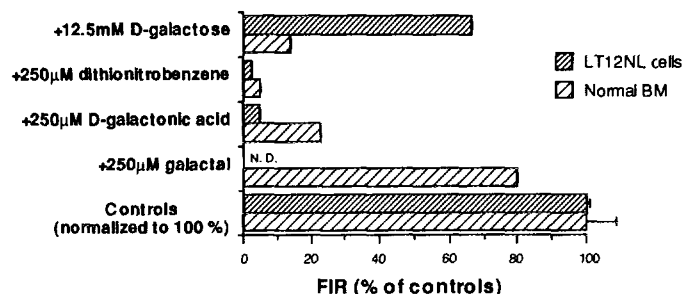

FIG. 3. Exploring the suppressive effect of galactose derivatives on lysosomal (▨) and *E. coli* (▨) β-galactosidase activity. Bars express percentages of respective control. Error bars express standard deviations of triplicate measurements.

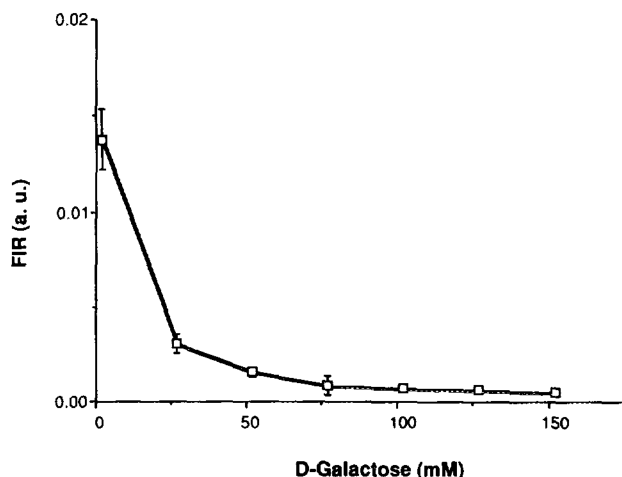

FIG. 4. Dose-effect relationship between D-galactose and fluorescence intensity increase rate (FIR) of lysates from 5×10^6 normal bone marrow cells. Error bars represent standard deviations of triplicate measurements.

on *E. coli* β-galactosidase vs lysosomal β-galactosidase. The compounds were tested in our kinetic MUG assay, using PBS as reaction buffer and lysates from 5×10^6 normal bone marrow cells and from 10^5 *LacZ*-marked cells as enzyme sources.

RESULTS

Determination of optimum pH. In end point MUG assays the reaction is stopped by shifting the pH of the reaction mixture to pH 12. This denatures the enzyme and increases the fluorescence efficiency of 4-MU, the fluorescent reaction product of MUG. A real time kinetic assay must be performed at physiological pH values. The optimum pH of *E. coli* β-galactosidase is at pH 7.0. The optimum fluorescence efficiency of 4-MU is at pH values above pH 9.4 (Fig. 1). Therefore, in a kinetic MUG assay the observed increase of fluorescence intensity is a resultant of decreasing enzyme activity at basic pH values and of increasing fluorescence efficiency of 4-MU. The combination of these effects led to an optimum pH for performing the assay at pH 8.0 (Fig. 2, open circles).

Suppression of background activity. Lysates from 5×10^6 bone marrow cells from healthy control rats show considerable background activity due to the presence of an endogenous β-galactosidase in the lysosomes. Fiering *et al.* (4) reported on the use of chloroquine for the suppression of endogenous β-galactosidase activity. Chloroquine is accumulated in intact lysosomes, which raises the intralysosomal pH and thereby reduces lysosomal β-galactosidase activity. Obviously, this works only in intact cells. To find a way to selectively suppress background β-galactosidase activity in lysates, we tested a number of substances that were reported as inhibitors of specific β-galactosidases (9). Of the substances tested (Fig. 3), galactal showed only a minor suppression of lysosomal β-galactosidase and was therefore not tested with *E. coli* β-galactosidase. Dithionitrobenzene and D-galactonic acid had their strongest suppressive effect on the *E. coli* β-galactosidase. Only D-galactose was seen to selectively suppress endogenous β-galactosidase activity. In a dose ranging experiment it was shown that a concentration of 100 mM D-galactose led to an almost saturated suppression of background activity (Fig. 4). This concentration was used in all further experiments.

Combined effect of D-galactose and pH. To investigate whether the use of D-galactose might have an influence on the optimum pH of the kinetic MUG assay, the pH was varied in the presence and in the absence of 100 mM D-galactose in the measurement of background activity (Fig. 5) and in the measurement of *E. coli* β-galactosidase activity from LT12NL15 cells (Fig. 2). In the pres-

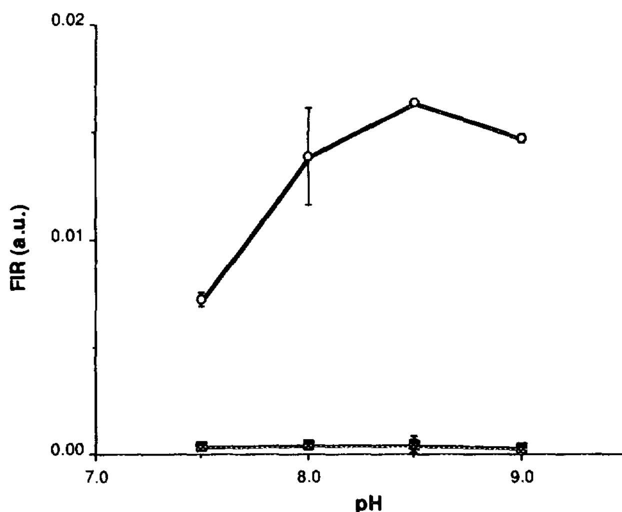

FIG. 5. Influence of the pH on fluorescence intensity increase rate (FIR) of lysates from 5×10^6 normal bone marrow cells, with (■) and without (○) 100 mM D-galactose. Measurements were done in duplicate.

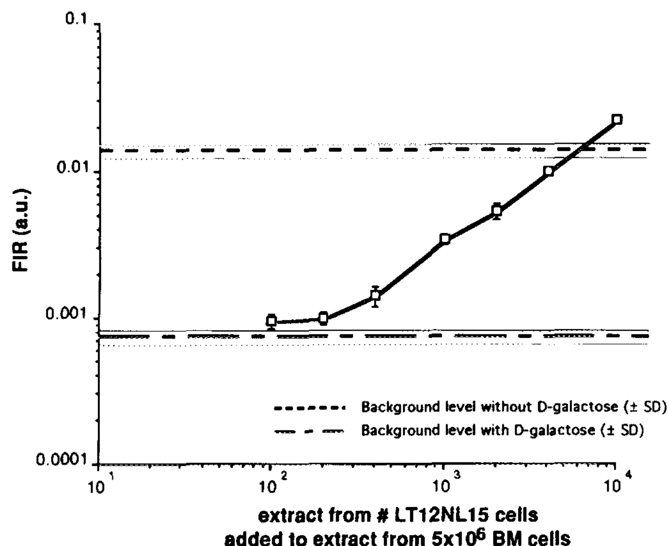

FIG. 6. Determining the lower detection limit of LT12NL15 cells in a background of 5×10^6 normal bone marrow cells. Error bars represent standard deviations of triplicate measurements.

ence of 100 mM D-galactose the activity of lysosomal β-galactosidase was suppressed to a very low level, independent of the pH. The suppression factor at pH 8.0 was 31.6.

The optimum pH of *E. coli* β-galactosidase was still at pH 7.5–8.0 and was, similar to that of lysosomal β-galactosidase, less pH sensitive in the presence of D-galactose. At pH 8.0 we measured a 4.7-fold suppression of *E. coli* β-galactosidase activity. Thus, the use of 100 mM D-galactose led to a 6.7-fold differential suppression of lysosomal β-galactosidase activity vs *E. coli* β-galactosidase activity.

Determining the detection level. After determining the optimum assay conditions we determined the lower detection level of LT12NL15 cells in lysates from 5×10^6 normal bone marrow cells (Fig. 6). When the lysate from 400 LT12NL15 cells was added, the FIR was raised to twice the background level, which is generally considered to be the lowest significant level in an assay. Above 400 LT12NL15 cells, the FIR was seen to increase linearly along with the number of cells added. Therefore, the lower detection limit of the assay was at 400 LT12NL15 cells in a background of 5×10^6 bone marrow cells.

DISCUSSION

The therapeutic regimens that are tested in the BNML model have a strong, but variable influence on the cellular composition of the bone marrow. Some of these treatments, such as total body irradiation, are known to induce strong autofluorescence in bone marrow cells. Therefore, we chose to develop a kinetic assay, in which the initial background fluorescence in the samples does not influence the measurements. We used MUG as a substrate, because fluorometric MUG assays are generally more sensitive than colorimetric assays using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. To optimize our assay, we first had to assess the effect of pH, because the pH influences three major components of the assay system: 4-MU fluorescence efficiency (Fig. 1.), lysosomal β-galactosidase activity, and *E. coli* β-galactosidase activity. Figure 2 shows that the optimum pH for measuring *E. coli* β-galactosidase activity alone is at pH 8.0, as a result of the combined effect of decreasing enzyme activity above its optimum pH of 7 and the increasing fluorescence efficiency of 4-MU. Surprisingly, lysosomal β-galactosidase activity is measured most efficiently at pH 8.5. This is probably due to the fact that so far above its activity optimum (pH 4.5), the activity of the lysosomal enzyme only slightly decreases with a rise in pH, whereas the fluorescence vs pH curve of 4-MU increases rather steeply at pH 8–9.

The sensitivity of a detection method is determined by the level of background activity. Suppression of background is therefore crucial to achieve improved detection sensitivity. Young *et al.* (10) described the use of heat treatment for the suppression of endogenous β-galactosidase activity in eukaryotic cell lines. Lysates of LT12NL15 cells, our *LacZ*-expressing cell line, showed strong reduction of activity after 60 min incubation at 50°C. This heat lability could be caused by the truncation of the first eight carboxy-terminal amino acids from the β-galactosidase produced by the marker gene (11) in comparison to purified bacterial enzyme. The endogenous β-galactosidase activity in bone marrow lysate was only slightly reduced by the heat treatment. Thus, in our system heat treatment did not result in background reduction (results not shown). Therefore, several substances that were reported in the literature as inhibitors of β-galactosidase activity were tested for their applicability as selective suppressors of lysosomal β-galactosidase activity. Apart from D-galactose, all the compounds that were tested were more effective in suppressing *LacZ* activity or did not have any considerable effect at all. Only D-galactose was seen to inhibit lysosomal β-galactosidase activity more than *LacZ* activity. After selecting 100 mM D-galactose as the standard concentration to work with (Fig. 4), we verified that the optimum pH for performing the assay in the presence of D-galactose was still at pH 8.0. At this pH, the addition of 100 mM D-galactose led to a 6.7-fold differential suppression of lysosomal β-galactosidase activity.

After optimizing the assay conditions, we determined the detection limit of our assay by mixing lysates of varying numbers of LT12NL15 cells with lysates of a fixed number of 5×10^6 normal bone marrow cells. At 400 LT12NL15 cells, the FIR is twice that of background, a level that is generally considered as the lowest detect-

able. This means that fewer than 500 leukemic cells per 5×10^6 normal bone marrow cells can be detected with this assay. This may not seem very sensitive when compared to, for example, the luminometric AMPGD assay described by Jain and Magrath (6), which can detect the β-galactosidase activity of a single *LacZ*-expressing cell. However, this assay, as indeed most assays described, was only optimized for measuring *E. coli* β-galactosidase expression in cell lines, not for large tissue samples containing a large amount of endogenous lysosomal background activity. When compared to other assays for the detection of rare leukemic cells, our MUG assay shows comparable or better sensitivity than other assays such as antibody staining and agar culture assays (1) and is less time consuming.

The use of D-galactose for the differential suppression of background activity may be generally useful in all *LacZ* assays to increase detection sensitivity not only in rat cells, but also in human and murine systems, where it has a comparable effect on background activity (data not shown). In histochemical staining assays using X-gal as a substrate for β-galactosidase, D-galactose can prevent background-containing cells from developing a "threshold" amount of staining, making such assays free of any background problems, so that every stained cell can be positively identified as a cell expressing the *LacZ* gene (data not shown).

In conclusion, we have optimized a kinetic fluorometric assay for the detection of *LacZ*-marked leukemic cells in the presence of large numbers of normal bone marrow cells. The assay was optimized by carefully defining the optimum pH of the assay and by using D-galactose, which causes a 6.7-fold differential suppression of mammalian lysosomal β-galactosidase activity. This led to a detection sensitivity of one leukemic cell per 10^4 normal bone marrow cells.

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