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Determination of lactate dehydrogenase isoenzymes in single lymphocytes from normal and leukemia cell lines

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Abstract

This work demonstrates that our previously developed technique for single-erythrocyte analysis by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) can be applied to study individual lymphocytes, with some modification in the cell lysing procedure. A tesla coil was shown to be capable of lysing the lymphocyte cells inside the capillary. The electromagnetic field induced by the tesla coil was believed to be responsible for breaking the cell membrane. The lactate dehydrogenase (LDH) isoenzyme activities and the relative ratios between different LDH isoenzymes were measured for normal lymphocytes as well as B-type and T-type acute lymphoblastic leukemia cells. Both the LDH activity and the isoenzyme ratios show large variations among individual cells. The former is expected due to variations in cell size. The latter implies that single-cell measurements are less useful than the average values over a cell population as markers for leukemia.

Keywords: Lactate dehydrogenase; Enzymes; Isoenzymes

1. Introduction

The timely diagnosis of cancer (or carcinoma) is very important for the treatment and even cure of the disease. It is well known that before morphological changes are detectable, cytochemical changes have long undergone gradual transformation. The concentrations of many molecular markers or biomarkers (e.g. growth factors, proteins, polyamines, DNA adducts, etc.) are constantly changing during carcinogenesis. Much effort therefore has been and will continuously be put into finding and defining potential molecular markers for different kinds of cancers [1–6]. Amongst these markers, the enzymes attracted much attention because they control the balance of cytochemicals and actively participate in the cell proliferation process. As investigated previously [3,4,7–9], enzymes might show different activity in tumor cells compared to that in normal cells, and some isoenzymes even showed different patterns.

Lactate dehydrogenase (LDH) has been found to be a very valuable enzyme in diagnosing different kinds of diseases, such as liver disease, myocardial infarction, etc. [10]. More significantly, many researchers noticed that both the LDH activity and the LDH isoenzyme patterns in serum are potential biomarkers .for different cancers [11,12] and leukemia [13]. In the sera collected from cancer patients, the LDH-2 form generally showed an elevated activity, even when the total LDH level was normal. However, the results were not very con-

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sistent among different groups of patients. The intracellular LDH activity and the ratio for isoenzyme activity in lymphoblastic leukemia cells were also quantified [14] from large amounts of lysed cells. The measured activity indicated that LDH-4 has a relatively higher activity while the total LDH activity is lower than that in the normal lymphocytes. Also, the LDH-4/LDH-2 activity ratio is higher in lymphocytic leukemia cells than in the normal lymphocytes. Furthermore, the investigation by Bottomley et al. [15] indicates the possibility of using LDH activity to classify leukemia.

Recently, capillary electrophoresis has become a powerful tool for studying intracellular components in different kinds of single cells [16]. By coupling to different detection methods, a large variety of intracellular components were probed, e.g. neurotransmitters and amino acids in neurons and adrenal medullary cells with electrochemical detection [17–19], and proteins and small ions in red blood cells with laser-induced fluorescence detection [20–24]. More recently, the catecholamines in single adrenal medullary cells were successfully determined with LIF detection by carefully controlling the pH [25]. The techniques developed for quantifying the LDH activity in single red blood cells [22] and the sensitivity achieved for monitoring the reactivity of single enzyme molecules [26] should enable us to quantify accurately the LDH isoenzyme activities for single lymphocytes. Therefore, the side-by-side analyses of normal lymphocytes and acute lymphoblastic leukemia cells will lend some insight about the difference in LDH activity between these cells and the possibility of using these as markers for leukemia.

2. Experimental

2.1. Instrumentation

The same home-made capillary electrophoresis apparatus as described before [22] was used throughout this study. Briefly, the mixture of 350 nm and 360 nm laser lines from an Ar ion laser (Model Innova 90-6, Coherent, Palo Alto, CA, USA) was used as the excitation source at a power of 100 mW. A 1-cm focal length quartz lens was used to focus the laser beam to the detection window on the capillary. Untreated fused-silica capillaries, 22 μm I.D. and 350 μm O.D. with total length of 75 cm and effective length of 55 cm, were used for separating the LDH isoenzymes (Sigma, St. Louis, MO, USA) and as the micro-reactor for carrying out the enzymatic reactions. To minimize the background from stray light, a 380-nm cut-off filter and a 465-nm interference filter (10 nm bandwidth) were used in front of the photomultiplier tube window. For separation and for driving the individual product zones through the capillary +30 kV was applied across the capillary.

2.2. Detection method

In the running buffer (20 m*M* phosphate, pH 7.4), 1 m*M* NAD^+ and 3 m*M* lactate were added as the substrates for the enzymatic reaction. The reaction direction was chosen as follows:

$$NAD^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{pyruvate} + NADH$$

which is opposite to the natural reaction direction. One of the major reasons is the low background associated with the low fluorescence efficiency of NAD^+. Another reason is to avoid the inhibiting effect of pyruvate at high concentration on LDH [27]. Since the average volume of lymphocytes is at the picoliter range and the LDH enzyme is one of the minor proteins, the total LDH amount is much less than the amount of substrates available for the catalyzed reaction. At these conditions (pseudo-first-order reaction), the amount of NADH formed during a given period of time at a fixed temperature is linearly proportional to the LDH amount, more precisely to LDH activity. Therefore, the LDH activity can be quantified by measuring the amount of NADH formed during the fixed incubation period. In this work, longer separation times (3 min) and incubation times (5 min) were used to achieve a better *S/N* ratio for the enzyme assay to improve the measurement accuracy.

2.3. Cell treatment and injection

Normal human lymphocytes, T-type and B-type lymphoblastic leukemia cells were purchased from

American Type Culture Collection (Rockville, MD, USA). The cell lines were analyzed as received without further culturing. Usually, the cell lines were used as soon as possible after we received them. If not analyzed immediately, the cells were stored at a frozen state. Before doing the single-cell experiments, the cell lines were first thawed. Then, the same washing procedure and injection process as before [22] were used to isolate the cells and inject a single cell into the capillary for analysis.

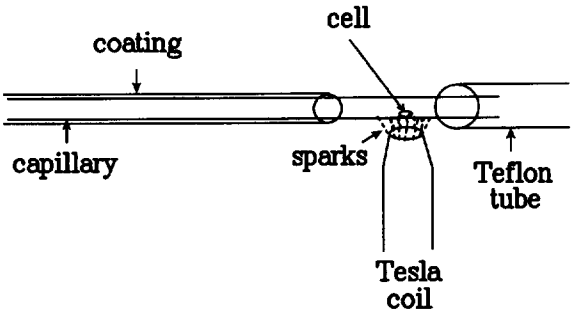

Fig. 1. Method for lysing single lymphocytes with a tesla coil.

3. Results and discussion

In the previous work on red blood cells [20–24], the running buffer readily lyses the cells in a short period of time (a few seconds) such that electrophoresis could be carried out immediately after cell injection. However, the lymphocytes are so rugged that special techniques are required to break them apart. For lysing a relatively large amount of cells, several methods had been demonstrated to be applicable, e.g. a mechanical homogenizer [12], an ultrasonic device [14], a quick freeze–thaw cycle [15], or chemical reagents [25]. When we tried to apply these techniques for lysing a single lymphocyte inside the capillary, there occurred some problems preventing these methods from being directly applicable.

From our experience, although the ultrasonic device can be used to lyse the cell inside the capillary, it also produces large disturbances, which dilute the intracellular components after their release in the buffer. This makes quantification very difficult. In the quick freeze–thaw method (with liquid N_2), lots of bubbles were produced inside the capillary after going through several cycles. The bubbles make it impossible to carry out capillary electrophoresis. Surfactants (e.g. SDS) were also tried for lysing the cell. These can lyse the cell in a very short period of time even at as low a concentration as 0.1%. Unfortunately, SDS also denatures the LDH isoenzymes, making it impossible to quantify the LDH activity.

Teissie [28] discussed that an external electric field could increase the cell membrane permeabilization and even break the cell membrane if the external voltage across the membrane is above ca. 200 mV. This prompted us to find a way to apply a voltage to induce an external electric field on the cell membrane for cell lysing. We tried to use a tesla coil to

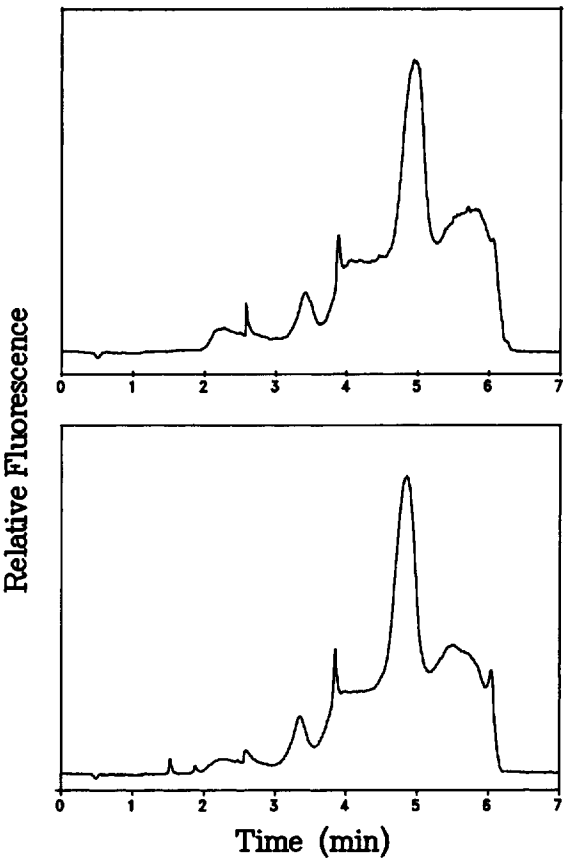

Fig. 2. Effect of the tesla-coil method on LDH activity. The conditions for on-capillary LDH assay of the standard mixture are described in the text. Bottom: with the tesla coil; top: without the tesla coil

induce an electric field and found that it was able to lyse the cell in a reasonable period of time (ca. 15–20 s). The lysing process is shown schematically in Fig. 1. After a single cell was injected into the capillary, the capillary was moved away from the microscope and fixed on top of a plastic rod. The capillary inlet was then covered with a short piece of teflon tubing to avoid introducing bubbles into the capillary. When the tip of the tesla coil touches the outside of the teflon tube, the induced electric field on the capillary wall passes on to the cell membrane. After the cell was lysed, the capillary was moved back to the buffer vial to initiate the on-column enzyme assay.

To investigate the effect of the tesla coil on the LDH activity, a LDH standard mixture (50 nI.U. each) was analyzed under conditions with and without applying the tesla coil after sample injection. The same conditions as used for single-cell analysis were used for the standard assay. As can be seen in Fig. 2, the LDH activity did not show noticeable differences between the experiments with and without the tesla coil treatment. The tesla coil is thus well suited for lysing individual lymphocytes and similar cells for analysis by CE.

Fig. 3 shows electropherograms from the analysis of the LDH standard mixture (a) and lysates of normal lymphocytes (b), and T-type (c) as well as B-type (d) acute lymphoblastic leukemia cells. The amounts injected correspond to about 10 cells. The LDH isoenzymes are nicely separated by electrophoresis for 3 min, as confirmed by the LDH standards. Both the migration times and the peak patterns indicate that the major LDH isoenzymes in lymphocytes are LDH-3 and LDH-4, which are quite

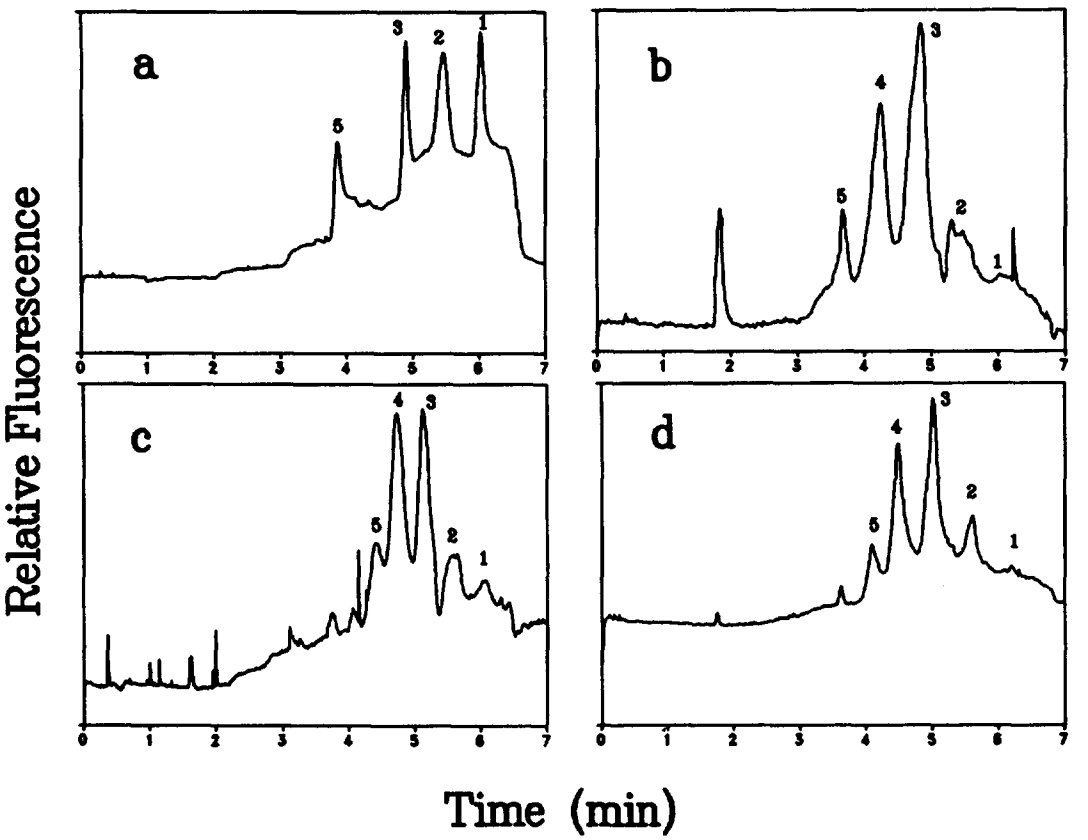

Fig. 3. Electropherograms of the analysis of the LDH standard mixture (a), lysed normal lymphocytes (b), lysed T-type (c) and lysed B-type (d) lymphoblastic leukemia cells.

different from the patterns in red blood cells where the major forms are LDH-1 and LDH-2. The distribution of LDH isoenzymes are organ-dependent [29]. The LDH isoenzyme abundance follows the order: LDH-3>LDH-4>LDH-5>LDH-2>LDH-1, which is different from the order reported in Ref. [27] (LDH-3>LDH-2>LDH-4>LDH-1>LDH-5).

In Fig. 4, electropherograms of a single-cell analysis for each cell line are shown. Usually, three or four peaks were observed for most of the individual cell analyses. In several experiments we observed five peaks, corresponding to the five LDH isoenzymes. The percentage of successful single-lymphocyte analyses as a fraction of all cell injections is about 40%, being substantially lower than that of red blood cell analyses (about 80%). The likely reason is related to the cell lysing process, which is not as gentle or as complete as the hypertonic lysing process. The tesla coil can sometimes cause a relatively large disturbance that moves the cell out of the capillary. At other times it may not produce enough shock to break the cell membrane. Also, since the tesla coil only breaks the cell membrane, the undissolved membrane debris may produce extra peaks. As we had observed earlier [22], the migration time changes after several cells are analyzed due to the adsorption of intracellular proteins and the cell membrane materials. We found that the capillary can be reconditioned by simply flushing it with running buffer and re-equilibrating for 30 min.

The size of the lymphocytes varied in a fairly large range, with diameters of 6–14 μm [30]. The size variations can be confirmed by using a 100× microscope objective during the process of single-cell injection. It is very difficult to pick up only cells of a uniform size to be injected for analysis. As we expected from the large size variations, the intracellular LDH activities showed quite large variations, as

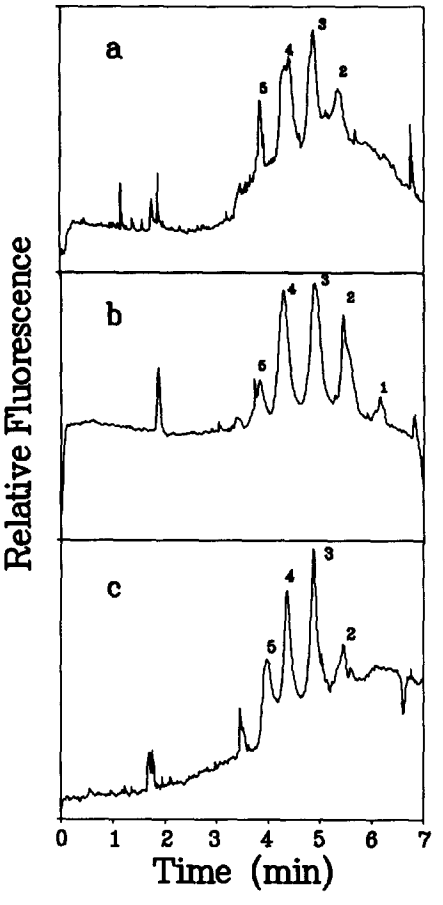

Fig. 4. Electropherograms of single-cell analysis: (a) normal lymphocyte; (b) T-type lymphoblastic cell; and (c) B-type lymphoblastic cell.

Table 1
Variations of activities in single-cell studies

	Mean	S.D. (%)
Normal cells		
LDH-1	1160	49
LDH-2	1350	82
LDH-3	3550	100
LDH-4	2620	76
LDH-5	1160	92
LDH-4/LDH-2	2.4	52
T-cells		
LDH-1	850	63
LDH-2	800	85
LDH-3	2950	84
LDH-4	2380	97
LDH-5	970	68
LDH-4/LDH-2	3.5	62
B-cells		
LDH-1	830	103
LDH-2	1070	166
LDH-3	3600	65
LDH-4	3000	57
LDH-5	1470	112
LDH-4/LDH-2	4.2	59

shown in Fig. 5 and Table 1. If a size-sorting method was used to put the lymphocytes into groups with uniform sizes, the absolute intracellular LDH activity would be more meaningful. The cell age might also be a big factor for the large activity variations because of the long life span of the lymphocytes [30]. The average total LDH activity for the normal lymphocytes is higher than that in both B-type and T-type lymphoblastic leukemia cells. From the cells studied, the average LDH activities are 6.3, 5.2 and 5.0 nI.U. respectively for the normal lymphocytes, T-type and B-type leukemia cells.

Even though there is large size variation (>10× in volume) among the cells, the ratio of different LDH isoenzymes might remain constant if the cells were at the same state of health and metabolic state. The LDH-4/LDH-2 ratio was observed to be elevated in the serum of different cancer patients [31,32], and in the leukemia cells from the analysis of cell lysates [14]. Therefore, we also examined this ratio for individual normal lymphocytes (Fig. 6a) and for individual acute lymphoblastic leukemia cells (Fig. 6b and c). During single-cell analysis, the on-capillary enzyme assay was calibrated frequently by using LDH standards. The variations were less than 15%, which indicated that the method is reliable and reproducible. On the average, the LDH-4/LDH-2 ratios for both T-type and B-type leukemia cells

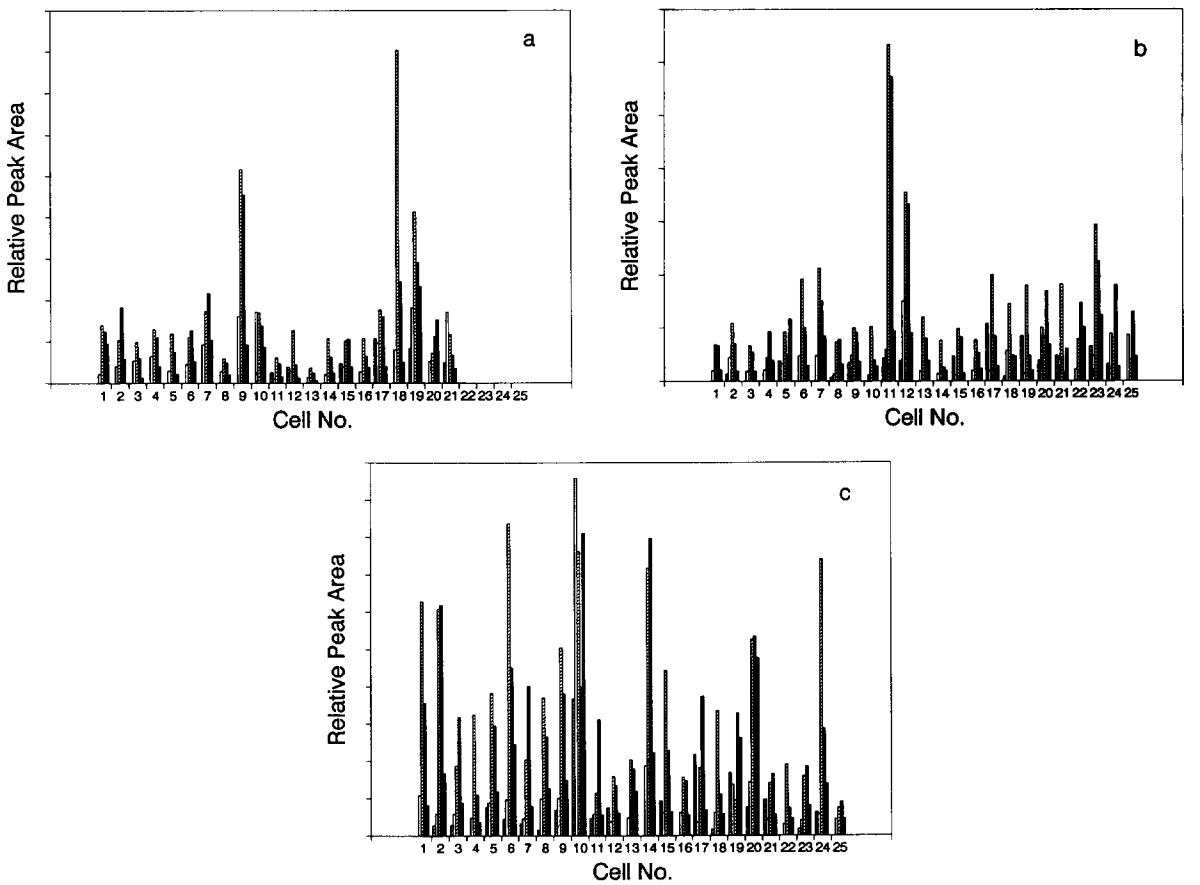

Fig. 5. Relative LDH activity in individual cells: (a) normal lymphocyte; (b) T-type lymphoblastic cell; and (c) B-type lymphoblastic cell. Cross-hatched, LDH-1; white, LDH-2; lightly hatched, LDH-3; heavily hatched, LDH-4; and black, LDH-5. The vertical scales are (a) 0–18 000; (b) 0–14 000; and (c) 0–10 000.

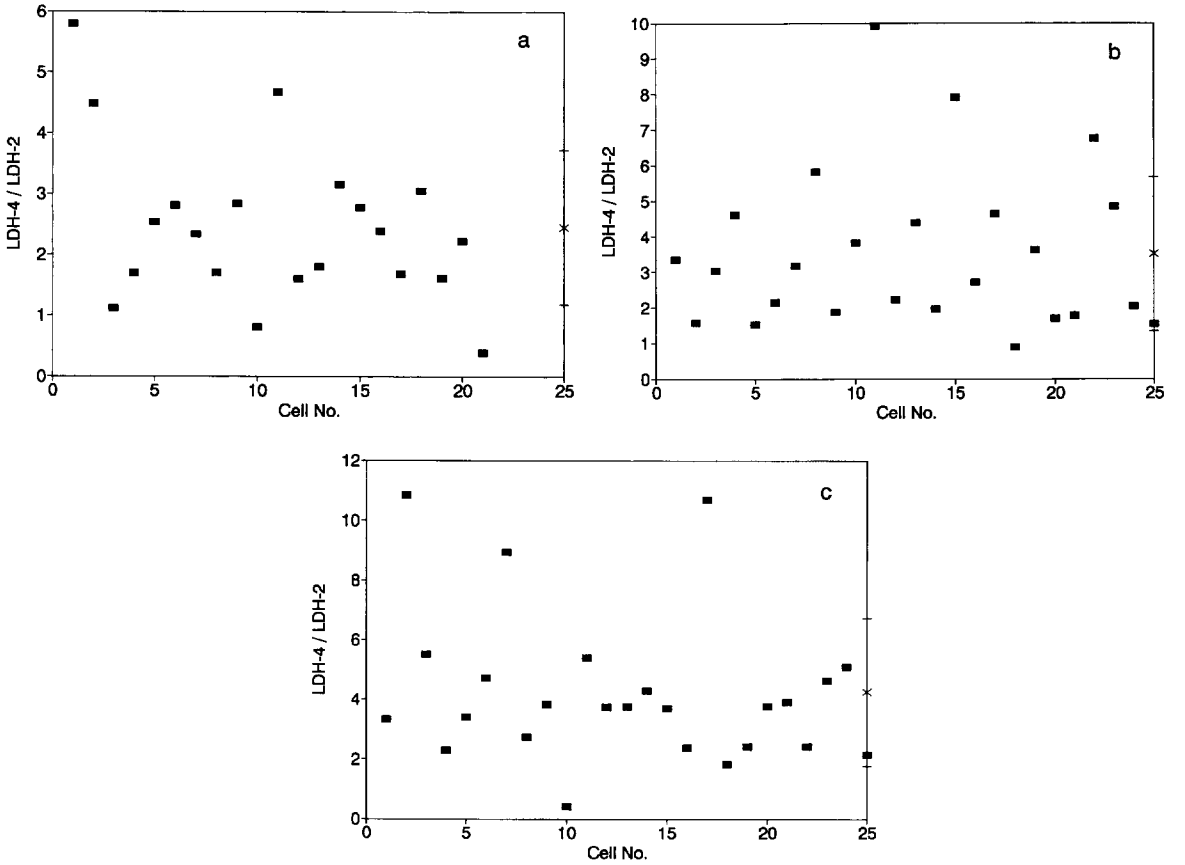

Fig. 6. LDH-4/LDH-2 ratios of individual cells for different cell lines: (a) normal lymphocyte; (b) T-type lymphoblastic cell; and (c) B-type lymphoblastic cell. To the right of each plot, the average ratio (cross) and the standard deviation (horizontal lines) are also shown.

were 43% and 73% higher, respectively, than that for the normal lymphocytes. The difference between T-type and B-type leukemia cells is also reasonable, as they have different functions and are formed in different organs [30]. Nevertheless, Fig. 6 shows that the observed elevation did not apply to every single cell in a given cell line. It appears that a reasonable number of cells need to be analyzed before any conclusions can be reached about their state of health. If many cells ($>10^6$) are available, the analysis of cell hemolysate will be advantageous in terms of providing an average value, for convenience, and for accuracy. Of course, if there is only a limited number of cells available for analysis, the single-cell technique here will stand in for providing statistical information about the intracellular components. The low detection limit offered by this technique and the small amount of material required for one measurement are still beneficial, particularly when applied to the routine monitoring of intracellular enzymes for less abundant entities, such as white blood cells.

Acknowledgments

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