ALTERATION OF MEMBRANE-ASSOCIATED ENZYMES IN DRUG-RESISTANT SUBLINES OF MOUSE LYMPHOBLASTOMA L5178Y CELLS

YOSHIKAZU SUGIMOTO, TOSHIO NISHIMURA, HIDEO SUZUKI and NOBUO TANAKA

Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan

(Received for publication May 6, 1981)

Activities of marker enzymes for various cell components were studied with extracts of adriamycin-, aclacinomycin A- and bleomycin-resistant cells and with partially purified plasma membrane fraction of aclacinomycin A-resistant cells, in comparison with those of the parental cells. Alkaline phosphodiesterase and Na^+-K^+-ATPase activities were observed to alter in the drug-resistant sublines, but other enzymes showed similar activities in the resistant cells to those in the parental cells. Alkaline phosphodiesterase activities in all the resistant sublines were higher than that in the parental cells. Na^+-K^+-ATPase activities of anthracycline-resistant sublines were lower and that in bleomycin-resistant cell line was higher than that of the parental cells. The adriamycin-resistant cells exhibited the same level of alkaline phosphodiesterase activity with the aclacinomycin A-resistant cells: V_{max} was the same with, and the affinity was twice stronger than the parental cells. The bleomycin-resistant cells showed *ca.* 30% V_{max} in comparison with the sensitive cells, and 17 fold higher affinity than the parental cells.

The current results, concerning changes of membrane-associated enzymes in drug-resistant sublines of L5178Y cells, support the assumption that the resistance is due to alteration of plasma membrane transport systems.

We have isolated adriamycin- and aclacinomycin A-resistant sublines of mouse lymphoblastoma L5178Y cells, and observed that the drug resistance is due to decreased influx and increased efflux of anthracyclines, resulting in lowered retention of the antibiotics in the cells[1~4]. A bleomycin-resistant subline has been also obtained, in which bleomycin-inactivating activity is not significantly altered[5]. These results suggest that the resistance is associated with some unknown changes in the plasma membrane. Based on this assumption, we have tried to isolate the plasma membrane fraction, using alkaline phosphodiesterase as a marker enzyme of cell membrane, and found unexpectedly that the specific activity of the enzyme is higher in the resistant cell sublines than in the parental cells. This finding has led us to further studies on various marker enzymes, which are used for fractionation of cell components, in bleomycin-, adriamycin- and aclacinomycin A-resistant sublines, in comparison with those in the parental cells. The results, showing alteration of plasma membrane in the resistant cells, are presented in this publication.

Materials and Methods

Cells

The sensitive and resistant cells of mouse lymphoblastoma L5178Y were cultured in Fischer's medium supplemented with 10% horse serum. The cells were washed with PBS buffer (NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g and KH_2PO_4 0.2 g per liter) or HEPES buffer (140 mM NaCl, 10 mM $MgCl_2$ and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), pH 7.4) for ATPase assay. The cell number was determined by a Coulter counter.

Enzyme Assay

The cells of 10^7/ml were ruptured in a Dounce homogenizer with a tight fitting pestle (type B), and the extract of 5×10^6 cells was used in each tube, containing 1 ml of reaction mixture or 3 ml for cytochrome C oxidase assay. Protein content was determined by the method of LOWRY *et al.*, using bovine serum albumin as a standard.

Alkaline phosphodiesterase (phosphodiesterase I) [EC 3.1.4.1] was assayed, using thymidine-5′-monophosphate-*p*-nitrophenylester as a substrate[6]. The reaction mixture, in 1 ml, contained: 50 mM tris-HCl, 5 mM $MgCl_2$ and 1 mM the substrate, pH 9.0. After incubation at 37°C for 15 minutes, the enzymic reaction was terminated by addition of 0.3 ml of trichloroacetic acid. The deproteinized supernatant was added to 0.4 ml of 2.5 N NaOH, and the optical density at 400 nm was compared with standard *p*-nitrophenol to determine the amount of substrate hydrolyzed.

Na^+-K^+-ATPase [EC 3.6.1.4] was measured by comparison of the following 2 systems, using ATP as a substrate. Total ATPase was determined in the reaction mixture (1 ml), containing: 140 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES, 14 mM KCl and 2 mM ATP, pH 7.1; and ouabain-resistant ATPase in the same mixture, in which 14 mM KCl was replaced by 1 mM ouabain. The difference between total ATPase and ouabain-resistant ATPase was taken as Na^+-K^+-ATPase. The amount of released Pi was measured by the procedure of BARTLETT[7].

5′-Nucleotidase [EC 3.1.3.5] was determined by the method of SUN and POOLE[8]. The assay system, in 1 ml, contained: 180 μM [2-^{3}H]AMP (0.5 μCi/ml), 12 mM $MgCl_2$ and 50 mM tris-HCl, pH 8.5. The mixture was incubated at 37°C for 10 minutes, and the reaction was stopped by addition of 0.2 ml of 250 mM $Ba(OH)_2$, followed by 0.2 ml of 250 mM $ZnSO_4$. The precipitate carrying unreacted AMP was separated by centrifugation at 3,000 rpm for 20 minutes. The radioactivity of the supernatant was determined in a liquid scintillation counter, using scintillation fluid, containing 30% Triton X-100.

Leucine aminopeptidase was assayed, using L-leucine-β-naphthylamide as a substrate[9]. The reaction mixture, in 1 ml, contained: 1 mM the substrate and 100 mM tris-HCl, pH 7.0. The mixture was incubated at 37°C for 15 minutes, and the reaction was terminated by addition of 0.1 ml of 50% trichloroacetic acid. The deproteinized supernatant of 0.8 ml was added to 1.0 ml of GBC solution, which consisted of 0.5 M acetate buffer, pH 4.1, 10% Tween 80 and 0.01% Fast Garnet GBC diazonium salt. The amount of substrate hydrolyzed was measured by OD_{525}.

Cytochrome C oxidase was assayed by the procedure of WARTON and TZAGOLOFF[10] with some modification. Cytochrome C was reduced by ascorbate in 10 mM phosphate buffer, pH 7.4, and ascorbate was removed by dialysis. The reaction mixture, in 3 ml, contained: 81 μM reduced cytochrome C and 20 mM phosphate buffer, pH 7.4. The incubation was carried out at 30°C, and increase of OD_{550} was measured.

β-Galactosidase was determined, using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. The reaction mixture, in 1 ml, contained: 2.5 mM the substrate and 50 mM pyridine-HCl, pH 5.0. After incubation at 37°C for 10 minutes, the reaction was stopped by addition of 0.1 ml of 50% trichloroacetic acid. The deproteinized supernatant of 0.8 ml was added to 0.25 ml of 0.5 N NaOH and 0.5 ml of 0.25 M Na_2CO_3-glycine buffer, pH 10.0. *o*-Nitrophenol released was measured by OD_{420}.

Isolation of Plasma Membrane

Plasma membrane was isolated by the procedure of BRUNETTE and TILL, using an aqueous two phase polymer system[11]. Stock solutions of the two phase system were prepared as follows: 40 g of 20% (w/w) dextran T500 in distilled water, 20.6 g of 30% (w/w) polyethyleneglycol 6000, 66.6 ml of 0.22 M sodium phosphate buffer, pH 6.5, 16 ml of 10 mM $ZnCl_2$ and 19.8 ml of distilled water were mixed in a separatory funnel, and allowed to settle for 24 hours in a cold room. The two phases were collected, and stored at 4°C.

L5178Y cells were washed twice with PBS and once with 0.15 M NaCl by centrifugation; and the cells of 10^9 were suspended in 40 ml of 1 mM $ZnCl_2$ for 15 minutes at room temperature, cooled in an ice bath for 5 minutes, and ruptured in a Teflon homogenizer: 200 strokes were required to disrupt more than 90% cells. The homogenate was centrifuged at 1,500 rpm for 15 minutes. The pellet was washed again by sedimentation, and used for separation in a two-phase system.

The cell pellet was suspended in 10 ml of the top phase, and then 10 ml of the bottom phase was added. The two phases were mixed and centrifuged at 8,500 rpm for 10 minutes. On completion of this centrifugation, the membrane was found at the interface of the two-phase system. The supernatant (*i.e.* the membrane and two-phase system) was poured into another tube, mixed and centrifuged at 8,500 rpm for 10 minutes. The material at the interface was then collected and diluted 4 fold with PBS. At this concentration, dextran T500 and polyethyleneglycol 6000 no longer formed two-phase. The interface was then sedimented at 5,000 rpm for 15 minutes. The pellet was suspended in PBS and used as the plasma membrane fraction.

Results

Enzymic Activities of Parental and Drug-resistant Sublines of L5178Y Cells

Activities of marker enzymes for various cell components were studied with extracts of adriamycin-, aclacinomycin A- and bleomycin-resistant cells, in comparison with those of the parental cells. Enzymes examined were: alkaline phosphodiesterase, Na^+-K^+-ATPase, leucine aminopeptidase and 5′-nucleotidase of plasma membrane, cytochrome C oxidase of mitochondria, β-galactosidase of lysosome, and ATPase of whole cell components. Activities of alkaline phosphodiesterase and Na^+-K^+-ATPase were found to alter in the drug-resistant sublines, but the other enzymes showed similar activities in the resistant cells to those in the parental cells (Table 1). Alkaline phosphodiesterase activity in the 3 resistant sublines was higher than that in the parental cells. Na^+-K^+-ATPase activity of adriamycin- and aclacinomycin A-resistant cell lines was less and that in the bleomycin-resistant line was higher than that of the parental cells.

Enzymic Activities of the Plasma Membrane Fraction

Enzymic activities of plasma membrane fraction, isolated from the aclacinomycin A-resistant cell subline, were compared with those from the parental cells. The results are presented in Table 2. The fraction of both cell lines exhibited significant activities of alkaline phosphodiesterase, Na^+-K^+-ATPase, and 5′-nucleotidase, but not that of cytochrome C oxidase, indicating that the isolated and partially purified fractions consisted of plasma membrane but did not contain mitochondria. The membrane preparation of the aclacinomycin A-resistant cells showed higher activity of alkaline phosphodiesterase,

Table 1. Activities of marker enzymes of parental and drug-resistant sublines of L5178Y cells.

Enzyme	Cell subline			
	Parental	Adriamycin-resistant	Aclacinomycin A-resistant	Bleomycin-resistant
Alkaline phosphodiesterase (pmole/min/mg protein)	10.5 (100)	13.8 (131)	14.1 (134)	23.6 (225)
Na^+-K^+-ATPase (μmole Pi/min/mg protein)	0.142 (100)	0.107 (75)	0.076 (53)	0.203 (143)
Leucine aminopeptidase (A_{525}/min/mg protein)	0.277 (100)	0.309 (111)	0.285 (103)	0.274 (99)
5′-Nucleotidase (cpm/min/mg protein)	1886 (100)	1964 (105)	1817 (97)	1980 (105)
Cytochrome C oxidase (A_{550}/min/mg protein)	680 (100)	669 (98)	714 (105)	694 (102)
β-Galactosidase (pmole/min/mg protein)	16.0 (100)	15.3 (96)	16.0 (100)	14.7 (92)
ATPase (μmole Pi/min/mg protein)	1.41 (100)	1.33 (94)	1.33 (94)	1.35 (96)

Fig. 1. LINEWEAVER-BURK plot of alkaline phosphodiesterase of parental and drug-resistant sublines of L5178Y cells.

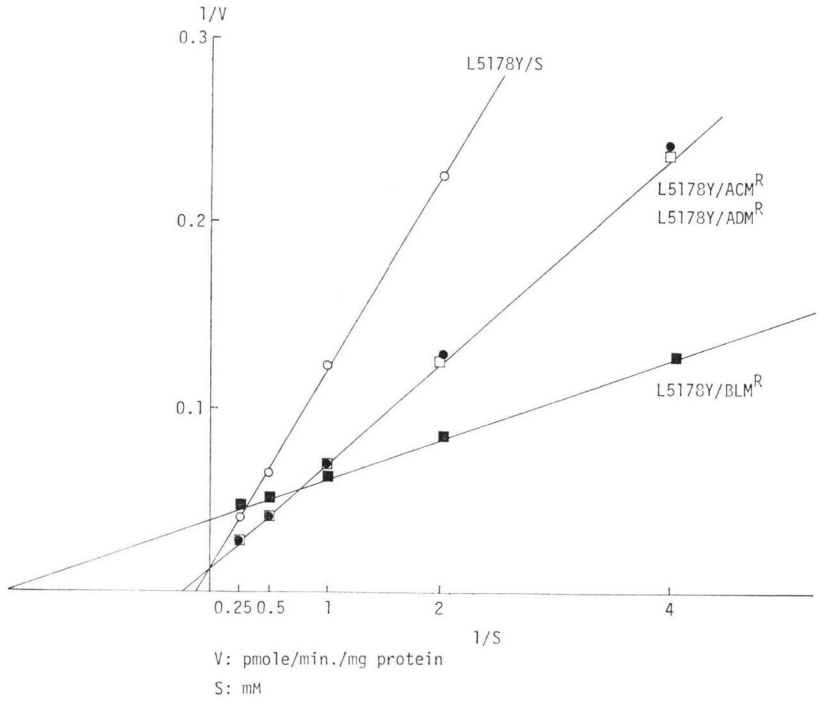

Table 2. Enzymic activities of the membrane fraction isolated from L5178Y cells.

Enzyme	Cell subline	
	Parental	Aclacinomycin A-resistant
Alkaline phosphodiesterase (pmole/min/mg protein)	31.5 (100)	54.7 (174)
Na^+-K^+-ATPase (μmole Pi/min/mg protein)	1.26 (100)	0.74 (59)
5′-Nucleotidase (cpm/min/mg protein)	12420 (100)	10690 (86)
Cytochrome C oxidase (A_{550}/min/mg protein)	0	6

Table 3. Activities of alkaline phosphodiesterase of parental and drug-resistant sublines of L5178Y cells.

Cell subline	Alkaline phosphodiesterase	
	V_{max}	K_m
Parental	91* (100)	10** (100)
Adriamycin-resistant	91 (100)	5 (50)
Aclacinomycin A-resistant	91 (100)	5 (50)
Bleomycin-resistant	26 (29)	0.6 (6)

* V_{max}: pmole/min/mg protein ** K_m: mM

and less activity of Na^+-K^+-ATPase than that of the parental cells. 5′-Nucleotidase activity was observed to be similar in both fractions. The revertant cell line exhibited enzymic activities similar to the parental cells (data are not shown).

Alkaline Phosphodiesterase of Parental and Drug-resistant Cells

LINEWEAVER-BURK plots of alkaline phosphodiesterase of parental and drug-resistant sublines of L5178Y cells are illustrated in Fig. 1, and V_{max} and K_m obtained from the plots are shown in Table 3. The adriamycin-resistant cells exhibited the same level of enzymic activity with the aclacinomycin A-resistant cells: *i.e.* V_{max} was the same with and the affinity was twice higher than the parental cells. The

bleomycin-resistant cells showed *ca.* 30% V_{max} in comparison with the parental cells, and about 17 fold higher affinity than the parental cells.

Discussion

The current experiments reveal that the anthracycline-resistant cell sublines possess higher activities of alkaline phosphodiesterase and less activities of Na^+-K^+-ATPase, and the bleomycin-resistant subline higher activities of both enzymes than the parental cells.

The partially purified plasma membrane fraction shows the same tendency of alterations of the enzymic activities as the cell lysate, suggesting that the changes are due to alterations of the enzymes *per se*. However, the altered enzymic activities can be also derived from changes of other proteins, which bind to the substrates, or changes of distribution of membrane components around the enzymes.

Since apparent ATPase activity of the whole cells in the resistant sublines do not differ from that in the parental cells, ATP-binding proteins may exist on similar levels in both cell lines, suggesting that alterations of Na^+-K^+-ATPase activity is attributed to changes of the enzyme itself, but not to those of other ATP-binding proteins. Of membrane-associated enzymes, alterations of activities are observed with alkaline phosphodiesterase and Na^+-K^+-ATPase, but not with 5′-nucleotidase and leucine aminopeptidase, suggesting that localization and/or amount of membrane components around the enzymes do not participate in the changes of enzymic activities.

Anthracycline-resistant and bleomycin-resistant cell lines, in which neither antibiotics shows significant cross resistance, exhibit different patterns of alterations of alkaline phosphodiesterase and Na^+-K^+-ATPase activities. The results suggest that the alterations of these membrane-associated enzymes are related to the mechanism of resistance.

As described in the accompanying paper[5], bleomycin-inactivating activity is low in the resistant cells and on a similar level to that in the parental cells. The current results show that the plasma membrane components are altered in the bleomycin-resistant cells, supporting the assumption that bleomycin resistance is due to alteration of the membrane transport system.

BOSMANN and KESSEL[12,13] have reported plasma membrane changes in glycoprotein and glycolipid content of actinomycin D-resistant cells, which are attributed to increased glycoprotein: glycosyl transferase activity and decreased glycosidase activity. Similarly BECK *et al.*[14] have reported altered surface membrane glycoproteins in vinblastine-resistant human leukemic cells, and suggested that the change is related to the resistance. However, the relationship of these results and ours remains open to discussion.

Acknowledgements

The current work was supported in part by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Dr. HAMAO UMEZAWA, Institute of Microbial Chemistry, for his generous advice and cooperations.

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