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On the Characteristics of Actinomycin D Resistance in L5178Y Cells[1]

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SUMMARY

A subline of the L5178Y murine leukemia was selected for resistance to actinomycin D by drug administration *in vivo*. The resistant line, L5178Y/D, had impaired capacity for uptake of actinomycin D *in vivo* and in culture but not under nongrowing conditions. Administration of the drug *in vivo* (50 μg/kg) or in culture (0.1 μg/ml) inhibited uridine incorporation into RNA by L5178Y but not by L5178Y/D. An altered cell surface glycoprotein fraction was found in L5178Y/D. Enzymatic studies showed that levels of glycoprotein transferases catalyzing formation of amino acid-sugar and sugar-sugar bonds were generally higher, while specific activities of glycosidases were generally lower in L5178Y/D. Alterations in membrane composition and conformation in the drug-resistant subline could account for the observed changes in actinomycin D permeability.

INTRODUCTION

The transplantable murine leukemias show a spectrum of responsiveness to actinomycin D (14, 26). Resistance to actinomycin D in murine leukemias (23), HeLa cells (18), and bacteria (29) is associated with permeability barriers which presumably prevent the drug-DNA interaction which is essential to the action of the drug (27). In this study, we have used the L5178Y cell line and an actinomycin D-resistant subline, L5178Y/D, to study consequences of selection for actinomycin D resistance on drug permeability and on enzymatic processes involved in metabolism of cell membranes.

MATERIALS AND METHODS

Tumor Cells. The L5178Y cell line was obtained from Dr. S. Okada, Department of Experimental Radiology, University of Rochester. The line was carried in CDF_1 mice by i.p. inoculation of 10^6 cells. An actinomycin D-resistant subline, L5178Y/D, was then derived by treatment of tumor-bearing animals on Days 2, 4, and 6 after transplant with 50 μg/kg of the drug. After 6 transplant generations, the subline was completely drug resistant and was then transplanted without further exposure to the drug.

Cell Number and Sizing. Cell numbers and size distribution of the cells were obtained by use of a Coulter Model B counter.

Chemicals. Nonlabeled actinomycin D was provided by Merck, Sharpe and Dohme, Rahway, N. J. The labeled drug (randomly tritiated, 4.2 mCi/mmole) was purchased from Schwarz BioResearch, Inc., Orangeberg, N. Y., and was provided by the Cancer Chemotherapy National Service Center. Uridine-2-^{14}C (10 mCi/mmole) and thymidine-2-^{14}C (25 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. HEPES[3] and TES were obtained from Calbiochem, Los Angeles, Calif. Substrates for glycosidase determinations (*p*-nitrophenyl glycosides) were obtained from Pierce Biochemicals, Rockford, Ill. Substrates for transferase determinations were UDP-galactose-^{14}C (200 Ci/mmole), UDP-glucose-^{14}C (200 mCi/mmole), and UDP-*N*-acetylglucosamine (40 mCi/mmole), purchased from New England Nuclear. GDP-fucose (110 mCi/mmole) was prepared as described (9). Papain was purchased from Worthington Biochemicals Corp., Freehold, N. J. Fischer's medium and horse serum were obtained from Grand Island Biological Co., Grand Island, N. Y.

Measurement of Uridine and Thymidine Incorporation into Nucleic Acids. Animals bearing L5178Y or L5178Y/D were used on Day 6 following transplant. A sample of the ascitic fluid (200 μl) was removed from the peritoneal cavity with a syringe and no. 26 needle, and the cells (10 to 20 mg) were collected by centrifugation. Actinomycin D (50 μg/kg) was then administered i.p. to each animal. Samples of ascitic fluid from each animal were then collected (22) at intervals after drug administration. Erythrocytes were eliminated by suspending each pellet in 0.2 ml of 0.2% NaCl solution for 10 sec; isotonicity was restored by addition of 0.2 ml of 1.6% NaCl solution. The cells were collected by centrifuga-

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[3]The abbreviations used are: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid; FHS, Fischer's medium containing 10% horse serum, but with 2.38 g/liter HEPES replacing $NaHCO_3$ (28); TES-E buffer, 75 mM TES (pH 7.2), 71 mM NaCl, 20 mM KCl, 1.3 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1 mM NaH_2PO_4; PSM, porcine submaxillary glycoprotein.

tion, the fluid was removed, and the wet weight of the pellet was determined. All centrifugations were for 30 sec at 500 × *g* in 10- x 30-mm siliconized glass tubes. Microscopic examination of cells stained with Wright's stain revealed a homogeneous population of murine lymphoblasts.

The pellets were suspended in buffered salts medium or modified (28) FHS composed of 1 part 10X Fischer's (17) medium (lacking $NaHCO_3$), 1 part horse serum, and 8 parts of 0.025 M HEPES buffer at pH 7.4. The pH of the medium was adjusted to pH 7.4 with HCl or NaOH. Labeled uridine-2-^{14}C (5 mM) or thymidine-2-^{14}C (2 mM) was added to the cell suspensions. Each incubation tube contained 1 ml of medium and 10 to 15 mg of cells, wet weight. After 30 min, incubations were terminated by chilling the suspensions, the cells were collected by centrifugation, the fluid was removed, and the pellets were suspended in 0.5 ml of cold 10% trichloracetic acid. The cells were collected by centrifugation, washed twice with 10% trichloracetic acid and once with water, and dissolved in 50 μl water + 450 μl of Nuclear-Chicago solubilizer. The clear solutions were diluted with 10 ml of a toluene-based phosphor for determination of radioactivity by liquid scintillation counting.

Alternatively, cells were collected and resuspended in TES-E buffer at 37°, and 0.5 μg/ml of actinomycin D was added. After 30 min, the cells were collected, washed once in FHS medium, and incubated in FHS medium with labeled precursors, as described above.

Permeability Studies. *In vivo* studies were carried out by treating animals bearing either L5178Y or L5178Y/D with labeled actinomycin D (diluted with carrier to a specific activity of 1 mCi/mmole), 50 μg/kg. At intervals following inoculation, cells were removed from the animals as described above, erythrocytes were eliminated, and 10-mg cell pellets were dissolved in 400-μl portions of Nuclear-Chicago solubilizer. After overnight digestion, the resulting colorless solutions were used for determination of radioactivity. A sample of the ascitic fluid was also taken for measurement of radioactivity; the cells were removed by centrifugation for 10 min at 5000 × *g*, and a 50-μl aliquot of the fluid was diluted with 400 μl of the solubilizer. After digestion, radioactivity was measured by liquid scintillation techniques.

In some cases, cells were collected from tumor-bearing animals and resuspended in the FHS or in TES-E buffer. Tritiated actinomycin D (final level, 0.1 μg/ml) was added to suspensions of 50 to 75 mg of cells/ml of medium. At intervals, 200-μl aliquots of the suspension were withdrawn and diluted with 0.8 ml of ice-cold 0.9% NaCl solution, and the cells were collected by centrifugation. The pellets were washed once in 0.9% NaCl solution; then they were solubilized with the Nuclear-Chicago solubilizer as described above. Since cell preparations used in these studies were thoroughly washed, no correction for trapped extracellular radioactivity was needed. Radioactivity was measured by liquid scintillation techniques.

Characterization of a Cell Surface Glycoprotein Fraction. Suspensions of L5178Y and L5178Y/D cells (2 g/20 ml) were digested with papain as described by Walborg *et al.* (31). The supernatant fluid from the papain-treated cells was deproteinized (24) with trichloracetic acid (final concentration, 5%), and the precipitate was removed by centrifugation. The fluid was dialyzed overnight against distilled water and concentrated under reduced pressure. The resulting solution was used for studies of levels of total carbohydrate by the anthrone method (15), fucose (16) and sialic acid (30). Suitable blanks were run by processing cells as outlined above, with papain omitted from the incubations. All data are presented with control values (cells incubated without papain) subtracted.

Cell Extraction for Glycoprotein Assay. The cells, harvested as described above, were extracted at 4° with 10 volumes of 0.1% Triton X-100 by 30 strokes in Ten Broeck homogenizer. Protein was determined by the method of Lowry *et al.* (25).

Glycosidase and Acid Phosphatase Assay. The assay for the glycosidases and acid phosphatase, previously described (3, 5, 11), is summarized below. Portions of the cell homogenate (100 μl each), containing about 0.9 mg of protein, were incubated for 1 hr at 37° with 6 μmoles of a *p*-nitrophenyl glycoside derivative or *p*-nitrophenyl phosphate and 50 μmoles of citrate buffer at pH 4.3, in a total volume of 1.1 ml.

The substrates used were *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-α-D-xylopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-L-fucopyranoside, *p*-nitrophenyl-β-L-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl phosphate. *p*-Nitrophenol was used as a standard. The reaction was terminated by addition of 2 ml of 0.4 M glycine-NaOH buffer at pH 10.5 The mixtures were centrifuged at 5000 × *g* for 10 min, and the absorbance of the *p*-nitrophenol released into the supernatant fluid was measured at 420 mμ. From these data and a standard *p*-nitrophenol concentration curve, the rates of hydrolysis were calculated in units of mμmoles/hr/mg protein. Determinations of activity were made at 10-min intervals; the reaction rates were linear for at least 4 hr. Two controls were run, with water replacing the enzyme or the *p*-nitrophenyl glycoside substrate. Each experiment was performed 5 to 9 times.

The enzymes studied were *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), α-D-glucosidase (EC 3.2.1.20), β-D-glucosidase (EC 3.2.1.21), α-D-galactosidase (EC 3.2.1.22), β-D-galactosidase (EC 3.2.1.23), β-L-fucosidase (EC 3.2.1.–), α-L-fucosidase (EC 3.2.1.–), β-D-xylosidase (EC 3.2.1.37), α-D-mannosidase (EC 3.2.1.24), *N*-acetyl-β-D-galactosaminidase (EC 3.2.1.–), and acid phosphatase (EC 3.1.3.2). Since the glycosidase activity was determined with *p*-nitrophenyl derivatives, the same activities may not obtain *in vivo* for macromolecular substrates such as glycoproteins, glycolipids, or polysaccharides (1).

Assay for Glycoprotein Transferases

"Endogenous activity" refers to enzyme activity found when distilled water was substituted for the acceptor in the

assay. "Exogenous activity" refers to the stimulation of enzyme activity above the endogenous which occurred when an exogenous acceptor was included in the assay. A brief description of each enzyme and the assay system used to determine its activity is given below.

Glycoprotein:Galactosyl Transferase. This enzyme transfers galactose from UDP-galactose to certain glycoprotein acceptors (23). The glycoprotein receptor was prepared from the α_1-acid glycoprotein by treatment with neuraminidase to remove sialic acid followed by treatment with snail β-galactosidase (Helicase) to remove galactose. It was concluded that galactose was transferred to terminal *N*-acetylglucosamine residues of the molecule. The complete incubation mixture (volume 0.155 ml) contained the following: 10 μl of 0.12 μM uniformly labeled UDP-galactose-^{14}C; 20 μl of 0.25 M $MnCl_2$; 15 μl of α_1-acid glycoprotein receptor, 0.075 mg protein; 50 μl of 0.1 M Tris buffer, pH 7.0; and 10 μl of 0.1% Triton X-100. After incubation at 25° for 45 min, the reaction was terminated, and the protein-bound radioactivity was determined as described above. The optimum pH for this enzyme is 7.0.

Glycoprotein:Fucosyl Transferases. These two highly specific enzymes, which transfer fucose from GDP-fucose-^{14}C to the appropriate glycoprotein acceptors (small-molecular-weight receptors are not active), were originally prepared from HeLa cells (9). One enzyme (the fetuin: fucosyl transferase) transfers fucose onto *N*-acetylglucosamine in an acceptor prepared from fetuin, and the other (PSM:fucosyl transferase) transfers fucose onto galactose in an acceptor prepared from PSM.

The fetuin acceptor for the fetuin:fucosyl enzyme was prepared from fetuin by treatment with neuraminidase (to remove sialic acid) followed by treatment with snail liver β-galactosidase (to remove galactose). The complete incubation mixture (volume, 0.135 ml) contained: 10 μl GDP-fucose-^{14}C (approximately 1 X 10^6 cpm), 50 μl of enzyme, 10 μl of 0.1% Triton X-100, 5 μl of 0.02 M glutathione, and 10 μl of 0.10 M $MgCl_2$. The pH of the assay mixture was adjusted to the enzyme optimum of 6.0 with 0.2 M Tris buffer. After incubation at 30° for 45 min, the reaction was terminated and the protein-bound radioactivity was determined as described above.

The acceptor from the PSM for the PSM:fucosyl enzyme was prepared by hydrolysis with 0.05 N H_2SO_4 at 85° for 90 min. The solution was neutralized, dialyzed, and lyophilized. The assay mixture contained (in a volume of 0.125 ml) 10 μl of GDP-fucose-^{14}C (approximately 1 X 10^6 cpm), 50 μl of the acceptor (0.1 mg protein), 50 μl of enzyme, 10 μl of 0.1% Triton X-100, and 5 μl of 0.02 M glutathione. The pH of the assay mixture was adjusted to enzyme optimum of 6.7 with 0.02 M Tris buffer. After incubation at 37° for 45 min, the reaction was terminated and the radioactivity was determined as described above.

Collagen:Glucosyl Transferase. Details of the specificity and assay system for this enzyme (6) and the distribution of the enzyme in HeLa cells (19) and fibroblasts transformed by oncogenic viruses (7) have been published. The enzyme transfers glucose from UDP-glucose onto galactose in collagen to form the glucose-galactose-hydroxylysine moiety of collagen. The incubation mixture contained 50 μl of acceptor (approximately 800 μg protein), prepared from guinea pig skin collagen, 10 μl of 0.1% Triton X-100, 10 μl of 0.25 M $MnCl_2$, 10 μl of UDP-glucose-^{14}C (approximately 40,000 cpm, 1.4 X 10^{10} mole), and 50 μl of the cell extract. The protein-bound radioactivity was determined as above. The time of incubation was 1 hr.

Collagen:Galactosyl Transferase. Details of the enzyme and assay system have been described (8) as has the distribution of the enzyme in HeLa cell membrane fractions and fibroblasts transformed by oncogenic viruses (2). The enzyme transfers galactose from UDP-galactose onto hydroxylysine in collagen to form the hydroxylysine-galactose linkage of collagen; the reaction is highly specific. The mixture, which was incubated for 1 hr at 37° contained: 50 μl of the cell extract (approximately 100 μg protein); 20 μl of acceptor preparation (approximately 900 μg protein); 10 μl of 0.1% Triton X-100; 10 μl of 0.1 M $MnCl_2$; 10 μl of UDP-galactose-^{14}C (approximately 40,000 cpm; 1.4 X 10^{-10} mole); and 10 μl of 0.1 M Tris buffer, pH 7.2; to a final volume of 110 μl. Protein-bound radioactivity was determined as described above.

Fetuin:*N*-Acetylglucosaminyl Transferase. Details of the specificity and assay for this enzyme have been described (4). The enzyme transfers *N*-acetylglucosamine from UDP-*N*-acetylglucosamine onto an acceptor of fetuin from which sialic acid, galactose, and *N*-acetylglucosamine have been removed, yielding a terminal mannose residue. Incubations were for 1 hr at 37°, in a mixture containing 10 μl of cell extract, 10 μl of 100 mM $MgCl_2$, 50 μl of the fetuin acceptor (200 μg of protein), and 10 μl of UDP-*N*-acetylglucosamine-^{14}C (0.25 μCi, 6 X 10^{-11} mole) in a volume of 80 μl.

RESULTS

Actinomycin D Responsiveness *in Vivo*. Treatment of animals bearing the L5178Y cell line with 50 μg/kg of drug from Day 1 to Day 7 after transplant prolonged survival by 200%. Actinomycin D was ineffective in prolonging survival of animals bearing L5178Y/D.

Cell Volumes. Sizing with the Coulter counter indicated no differences in cell size or cell volume when L5178Y and L5178Y/D were compared.

Effects of Actinomycin D on Nucleic Acid Synthesis. Administration of 50 μg/kg of drug to animals bearing L5178Y led to an inhibition of uridine incorporation and a stimulation of thymidine incorporation into nucleic acids. For data shown here, animals were used on Day 6 after transplant, and the cells were isolated 90 min after drug administration. Incorporation was measured *in vitro* as described under "Materials and Methods." Drug administration to animals bearing the L5178Y/D cell line, under similar conditions, did not significantly affect precursor incorporation into nucleic acids (Chart 1). These results could be duplicated, with small variations, with the use of cells isolated 1 to 24 hr after drug administration. The data shown represent typical values; because of variation in

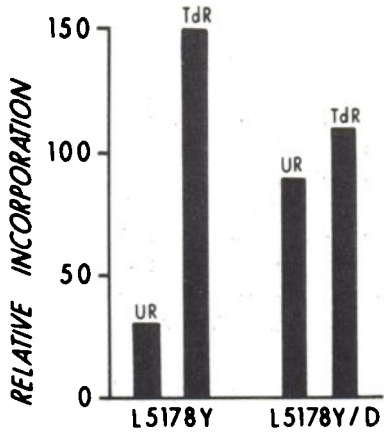

Chart 1. Effects of administration of actinomycin D *in vivo* on precursor incorporation into nucleic acids. Tumor-bearing animals were treated with 50 μg/kg of the drug, the cells were isolated 90 min later, and incorporation of uridine (*UR*) and thymidine (*TdR*) was measured as described in the text. For this typical experiment, 100% values correspond to 2.5 mμmoles of uridine and 2.3 mμmoles of thymidine/g cells, wet weight/15 min.

volume of ascitic fluid, a variation of ±20% is generally encountered among animals tested. Although no estimate of nucleotide pool sizes was made, we did find, in other studies, that the apparent stimulation by actinomycin D of thymidine incorporation into nucleic acids was accompanied by a corresponding increase in thymidine kinase levels in L5178Y cells.

The data of Chart 2 were obtained with L5178Y and L5178Y/D cells exposed to 0.5 μg/ml of actinomycin D in TES-E buffer. After 30 min, the cells were collected, transferred to different tubes, and incubated with labeled uridine or thymidine as described under "Materials and Methods."

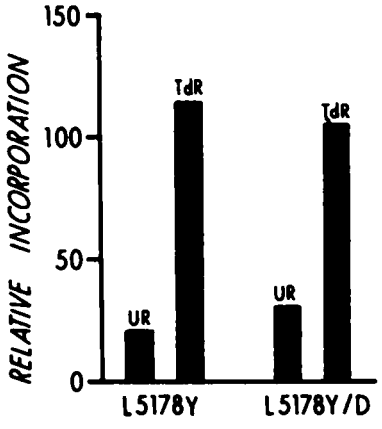

Chart 2. Effects of actinomycin D *in vitro* on precursor incorporation into nucleic acids. Freshly isolated cells were incubated with 0.5 μg/ml of drug in TES-E medium for 30 min. The cells were then incubated with labeled precursors in FHS medium as described in the text. For this typical experiment, control values (100%) correspond to 2.1 mμmoles of thymidine (*TdR*) and 1.9 mμmoles of uridine (*UR*)/g cells, wet weight/15 min.

Inhibition of uridine incorporation into nucleic acids was observed in both cell lines, but no significant stimulation of thymidine incorporation was observed. Data from a typical experiment are shown; these results could be duplicated within ±10% in other experiments.

Uptake of Actinomycin D-^{3}H. Animals bearing L5178Y or L5178Y/D were used on Day 6 after transplant. Each animal received 1 μg of labeled drug (1 mCi/mmole). After 30 min, the drug level in the cell-free ascitic fluid was 0.03 to 0.05 μg/ml. The drug level in the L5178Y cells had reached 0.3 to 0.5 μg/g, wet cells, representing an apparent distribution ratio of 6 to 10. In contrast, the L5178Y/D cells took up only 0.015 to 0.02 μg/g, wet cells, of the drug in 30 min, yielding a drug distribution ratio of less than 0.5. These data are summarized in Table 1.

Table 1

Uptake of labeled actinomycin D by L5178Y and L5178Y/D cells in vivo 30 min after inoculation with 50 μg/kg of the drug

Results of 3 typical experiments are shown.

Cell line	Level of actinomycin D-^{3}H: In fluid (μg/ml)	In cells[b] (μg/g)	Distribution ratio[a]
L5178Y	0.03	0.32	10.7
	0.045	0.36	8.0
	0.05	0.48	9.6
L5178Y/D	0.4	0.015	0.38
	0.035	0.02	0.57
	0.048	0.023	0.48

[a] Apparent distribution ratio of drug between wet cells and fluid.
[b] Units are in terms of drug uptake per g of wet cell.

For *in vitro* studies, the cells were removed from animals on Day 6 after transplant and suspended in FHS or TES-E medium. For experiments shown here, the drug level was 0.5 μg/ml. The extent of drug uptake was proportional to the drug level in the medium over the range 0.01 to 5 μg/ml. Labeled drug was taken up equally well by L5178Y or L5178Y/D cells in TES-E medium. In FHS medium, the L5178Y/D cells demonstrated the impairment in drug uptake also seen *in vivo*. These data are shown in Chart 3. In other studies, we found that suspension of L5178Y/D cells in FHS medium for 1 to 2 hr caused the barrier to drug uptake to disappear. This finding is probably related to the growth requirements of the L5178Y/D line which are not met by FHS. We have been unable to grow the L5178Y/D line in either FHS (28) or in the medium made up according to the original formula (17).

We previously reported (23) actinomycin D uptake by L1210 cells to be a temperature-sensitive phenomenon. In the present study, we found that lowering the incubation temperature to 0° decreased drug accumulation by both cell lines by 75% over a 30-min interval. Because of the extensive binding of actinomycin D to cell components (17), it is difficult to interpret the present data solely in terms of

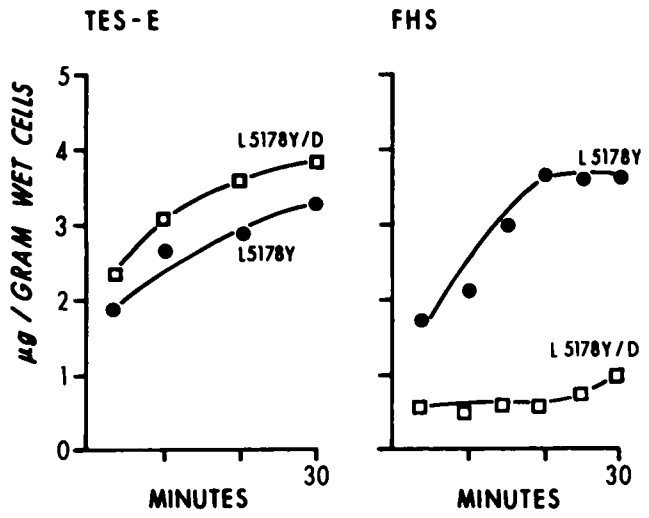

Chart 3. Accumulation of actinomycin D by L5178Y and L5178Y/D cells. Cells were suspended at 37° in buffered salts medium (TES-E) or modified FHS containing 0.5 μg/ml of labeled drug. Drug accumulation is shown in terms of μg of drug taken up per g of wet cells. These data are from a typical experiment; subsequent experiments varied from these data by less than ±10%.

transport. The results do show that an impairment to actinomycin D accumulation can be demonstrated in growing L5178Y/D cells.

Characteristics of Papain Digests of Cell Surfaces. Results of assays for carbohydrate in macromolecules released by papain from L5178Y and L5178Y/D cells are shown in Table 2, along with results of analyses of the isolated fraction for fucose and sialic acid. The drug-resistant line contained more carbohydrate, fucose, and sialic acid, in papain-sensitive linkages, than did the parent L5178Y line.

Glycosidase Activities. The levels of glycosidases found in L5178Y cells grown in mice were similar to data obtained from cells grown in culture (5). The L5178Y cells are characterized by the absence of α- and β-fucosidase and high activities of β-*N*-acetylglycosaminidase and acid phosphatase. In each assay for glycosidase activity, the L5178Y/D line had lower activity than did L5178Y. This was most striking for: (*a*) β-*N*-acetylglucosaminidase (260 mμmoles/hr/mg protein in L5178Y; 100 mμmoles/hr/mg protein in L5178Y/D); (*b*) α-glucosidase, which had an activity in L5178Y of 10 mμmoles/hr/mg protein and in L5178Y/D of 3 mμmoles/hr/mg protein (Table 3). Acid phosphatase activity in both L5178Y/D and L5178Y cell lines was equal (about 207 mμmoles/hr/mg protein), which indicated that the decreased glycosidase activity was not indicative of a general decrease in levels of lysosomal enzymes.

Glycoprotein Transferases. Measurements of endogenous and exogenous transfer of monosaccharides from nucleotide diphosphate monosaccharide precursors indicated that the L5178Y/D cells had higher activity of membrane transferases and secreted transferases (Table 4). The differences in transferase activity between L5178Y and L5178Y/D were most striking when we compared endogenous transfer of glucose (collagen:glucosyl assay) and exogenous transfer of fucose onto fetuin and PSM acceptors.

Table 2

Composition of macromolecular species released by papain digestion
Each sample represents material obtained from 1 g of wet cells. Papain digestion and assays were carried out as described under "Materials and Methods."

Cell line	Anthrone[a] (μg/g)	Fucose (μg/g)	Sialic acid (μg/g)
L5178Y	550	150	140
L5178Y/D	1200	284	560

[a]Reported as μg glucose equivalents/g of wet cells.

Table 3

Glycosidase and acid phosphatase activity of L5178Y and L5178Y/D cells
Data given as mμmoles/hr/mg protein. Means ± 1 S.D. Cells were extracted and assays performed as given under "Materials and Methods."

Enzymes	L5178Y	L5178Y/D
α-Fucosidase	0	0
β-Fucosidase	0	0
β-Xylosidase	2 ± 0.1	1 ± 0.2
α-Mannosidase	9 ± 3	4 ± 1
α-Glucosidase	10 ± 1	3 ± 1
β-Glucosidase	14 ± 1	8 ± 1
α-Galactosidase	28 ± 2	18 ± 2
β-Galactosidase	74 ± 4	31 ± 1
β-*N*-Acetylgalactosaminidase	20 ± 2	10 ± 1
β-*N*-Acetylglucosaminidase	260 ± 11	100 ± 4
Acid phosphatase	206 ± 10	209 ± 8

DISCUSSION

In a previous study (25), we found that cell lines differing widely in responsiveness to actinomycin D accumulated similar amounts of the drug *in vitro* under nongrowing conditions. Accumulation of actinomycin D *in vivo* by different cell lines varied considerably; significant retention after 24 hr was found only in drug-responsive cell lines. Other investigators have related actinomycin D resistance to barriers to drug uptake by growing cells (18, 27, 29).

In the present study, a drug-resistant subline of the L5178Y leukemia was developed by exposure of tumor-bearing animals to actinomycin D. The resistant subline, L5178Y/D, unlike the parent L5178Y line, could not be cultured in Fischer's medium, indicating that nutritional requirements of the cell had been altered as a result of the selection. The capacity of L5178Y cells to incorporate uridine and thymidine into nucleic acids was markedly affected by actinomycin D administered *in vivo* or in culture. Incorporation of uridine was inhibited, while that of thymidine was stimulated. Such findings have been described before (27). Incorporation of these precursors into nucleic acids by L5178Y/D *in vivo* was not much affected by actinomycin D. These differences, which could be observed

Table 4

Activity of glycoprotein transferases in L5178Y/D cells

Data are given as cpm/mg protein (mean ± 1 S.D.) Cells were extracted and assays were performed as given under "Materials and Methods." "Endogenous activity" refers to the capacity of cell extracts to incorporate a specified sugar into a glycoprotein with endogenous glycoprotein acceptors. "Exogenous activity" represents the additional incorporation achieved when an excess of glycoprotein acceptor was added. The former is therefore an index of acceptor levels in the homogenate; the latter is an index of enzymatic capacity of the homogenate.

	Endogenous activity		Exogenous activity	
Transferase	L5178Y	L5178Y/D	L5178Y	L5178Y/D
Collagen:glucosyl	320 ± 14	78 ± 14	585 ± 11	690 ± 31
Collagen:galactosyl	2130 ± 106	3619 ± 49	1870 ± 42	2410 ± 114
Glycoprotein:galactosyl	770 ± 17	1470 ± 21	106 ± 16	167 ± 10
Fetuin:fucosyl	680 ± 16	1111 ± 36	2493 ± 21	3610 ± 69
PSM:fucosyl	440 ± 14	972 ± 18	1211 ± 40	2206 ± 46
Fetuin:*N*-acetylglucosaminyl	239 ± 19	260 ± 7	217 ± 9	300 ± 11

in vivo, disappeared when stationary cultures of both cell lines were examined. Uptake of labeled actinomycin D was impaired in L5178Y/D *in vivo* or shortly after transfer of cells to culture. The data suggest that a facilitated, temperature-sensitive uptake process might be absent from growing L5178Y/D cells. However, the presence of a temperature-insensitive barrier to drug uptake in L5178Y/D is not ruled out. Drug uptake in FHS medium over the shortest times measured (3 min) was appreciably different in the two cell lines, which suggests possible differences in surface adsorption of drug.

As a result of our studies on comparisons between cell surfaces of L5178Y and L5178Y/D, we find that alterations in the composition and function of the cell membrane accompanied selection for actinomycin D resistance. The L5178Y/D cell line had markedly different levels of carbohydrate bound to the cell surface via papain-sensitive linkages (Table 2). These carbohydrates were found to contain fucose and sialic acid, both of which are normal components of glycoproteins. We have measured enzymes responsible for glycoprotein formation and degradation. The data indicate a general enhancement of levels of enzymes involved in synthesis and a decrease in the degradative glycosidases in L5178Y/D.

Levels of transferases responsible for both membrane and secreted glycoproteins are elevated in L5178Y/D. This situation is therefore different from examples of oncogenic virus-transformed cells that demonstrate elevated levels of membrane glycoprotein transferase (10) but reduced levels of secreted glycoprotein transferases (7). Glycosidase activities were generally increased in the virus-transformed cells (3, 12).

Actinomycin D-resistant cells are usually cross-resistant to daunomycin (24), vincristine, and certain phthalanilides (13, 14). Phthalanilide resistance has been related to accelerated exodus of drug from cells as a drug-lipid complex (32). Barriers to uptake of daunomycin (21) and phthalanilides (32) could not be demonstrated in stationary cells. Resistant and responsive cell lines were both sensitive to inhibition of RNA synthesis by actinomycin D or by daunomycin when in the stationary phase (20). These data suggest that alterations in cell surface membrane properties might be responsible for maintenance of drug permeability barriers in L5178Y/D and in certain other drug-resistant cells and that such alterations can be maintained only in growing cells.

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