Selection of Tunicamycin-resistant Chinese Hamster Ovary Cells with Increased N-Acetylglucosaminyltransferase Activity

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ABSTRACT   Chinese hamster ovary (CHO) cells resistant to the antibiotic tunicamycin (TM) have been isolated by a stepwise selection procedure with progressive increments of TM added to the medium. TM inhibits asparagine-linked glycoprotein biosynthesis by blocking the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to the lipid carrier. The TM-resistant cells exhibited a 200-fold increase in their LD_{50} for TM and were morphologically distinct from the parental cells. The rate of asparagine-linked glycoprotein biosynthesis was the same for wild-type and TM-resistant cells. Membrane preparations from TM-resistant cells cultured for 16 d in the absence of TM had a 15-fold increase in the specific activity of the UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-phosphate transferase as compared to membranes of wild-type cells. The products of the in vitro assay were N-acetylglucosaminylpyrophosphoryl-lipid and N,N'-diacetylchitobiosylpyrophosphoryl-lipid for membranes from both TM-resistant and wild-type cells. The transferase activity present in membrane preparations from wild-type or TM-resistant cells was inhibited by comparable levels of TM. The data presented are consistent with overproduction of enzyme as the mechanism of resistance in these variant CHO cells.

The biosynthesis of the oligosaccharide moiety of asparagine-linked glycoproteins involves a lipid carrier, dolichol phosphate, and a complex sequence of steps (1–3). One approach to delineating the pathway and its regulatory sites is to reconstitute the system using purified enzymes. Since the enzymes responsible for these reactions are membrane-associated and present in small amounts, purification would be facilitated by the generation of cell lines which overproduce these enzymes.

Overproduction of enzymes required for growth has been accomplished in certain mammalian cell lines by exposing the cells to increasing concentrations of potent, specific inhibitors of these enzymes (4–11), suggesting that overproduction may be a common mechanism of resistance to such inhibitors. In at least two cases, gene amplification has been demonstrated as the mechanism of overproduction (12, 13).

Tunicamycin (TM) is a tight-binding inhibitor of the first reaction in the dolichol-mediated pathway (14), and is cytotoxic to mammalian cells. This nucleoside antibiotic, isolated from *Streptomyces lysosuperificus* by Takatsuki et al. (15), inhibits the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate (14, 16–18). The

gross structure of TM has been determined (19); it is a bisubstrate analog which resembles UDP-N-acetylglucosamine and dolichol phosphate. TM, therefore, appeared to be a potential inhibitor for use in selection of mammalian cells which overproduce the N-acetylglucosamine-1-phosphate transferase. Here we present the selection and preliminary characterization of Chinese hamster ovary (CHO) cells which exhibit an increased resistance to TM.

MATERIALS AND METHODS

Chemicals and Radiochemicals

Mannose, N-acetylglucosamine, dextran (4×10^4 daltons), and chitin were purchased from Sigma Chemical Co. (St. Louis, MO). Dolichol phosphate was obtained from Calbiochem-Behring Corp. (La Jolla, CA). TM was a gift from Dr. J. B. Douros, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. D-[2-^{3}H]Mannose (13.2 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). UDP-N-acetyl-D-[^{3}H]glucosamine (6.6 Ci/mmol) was either purchased from New England Nuclear (Boston, MA) or synthesized as described by Owada and Neufeld (20). [^{3}H]Glucosylphosphoryldolichol, [^{14}C]mannosylphosphoryldolichol, and [^{3}H]oligosaccharide-lipid were prepared as described (21).

Analytical Methods

Dolichol phosphate concentrations were determined by analysis of lipid-soluble phosphate (22). Protein was determined by the method of Peterson (23) (for samples containing deoxycholate) or by the method of Lowry et al. (24) using bovine serum albumin (Sigma Chemical Co.) as the standard. In the latter case, particulate samples were diluted 1:12 with 1% SDS before the protein determination. Radioactivity was measured by liquid scintillation counting in Liquiscint (National Diagnostics, Somerville, NJ) as previously described (21). The elution positions of various standards on the gel filtration column were determined by one of the following methods: anthrone for carbohydrate (25), Morgan-Elson for N-acetylamino sugars (26), and Park-Johnson for reducing sugars (27). Polymers of N-acetylglucosamine were prepared from chitin as previously described (21).

Cells

Wild-type and TM-resistant CHO cells were routinely grown at 34°C in minimal essential medium (MEM), alpha-modified (K. C. Biologicals, Lenexa, KS) supplemented with 5% fetal calf serum (FCS) (Armour Pharmaceuticals Co., Kankakee, IL) attached to flasks or dishes obtained from Corning Glass Works (Corning, NY) or Falcon Division of Becton, Dickinson & Co. (Oxnard, CA) in a 5% CO_2 forced hot air incubator (Wedco, Inc., Silver Spring, MD). Cultures were periodically checked for mycoplasma (28) and found to be negative in all cases. After the final selection step, the TM-resistant cells were maintained in the medium described above supplemented with 27 μg TM/ml.

Selection of TM-resistant Cells

TM-resistant cells were selected by growing 1×10^6 wild-type CHO cells (WTB, see reference 21) in MEM containing 10% FCS (MEM-S) and 0.9 μg TM/ml. The surviving cells were maintained in this medium until the cells reached confluency. When necessary, colonies were treated with trypsin in phosphate-buffered saline, allowed to reattach to the original flask, and grown to confluency. Next, these cells were treated with 0.1% trypsin, and 5×10^5 cells from this population were seeded into another flask containing MEM-S. After one doubling, the cells were re-fed with MEM-S containing 2.9 μg TM/ml. The surviving cells were grown to confluency. This stepwise procedure was continued, sequentially increasing the concentration of TM to 9, 15, and finally 27 μg TM/ml. The FCS concentration was reduced to 5% after the final selection step. The survival rate at each selection step was ~0.01%.

Mannose Incorporated In Vivo

Wild-type and TM-resistant cells (~6.5 × 10⁵ cells/60-mm dish) were labeled in vivo with D-[2-³H]mannose (15 μCi/ml; 2 ml) for 1, 2, and 4 h in alpha-modified MEM without glucose (K. C. Biologicals) containing 5% dialysed FCS, 100 μM mannose, 5 mM pyruvate, and 15 mM tricine at pH 7.4. One group of wild-type cells was treated with 1 μg TM/ml for 3 h before, and during, the labeling period. Incorporation of mannose into the cells was terminated by rinsing the cultures several times with phosphate-buffered saline, pH 7.4, followed by solubilization of the cells in 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium azide, 0.5 mM phenyl-methylsulfonylfluoride, and 10 KIU/ml Trasylol. The amount of protein in each sample was determined (23). In addition, aliquots were precipitated in 5 vol cold 10% TCA and transferred to glass-fiber filters (GF/C; Whatman Inc., Clifton, NJ). The filters were then rinsed sequentially with cold 10% TCA, chloroform:methanol (2:1, vol/vol), chloroform:methanol:water (10:10:3, by vol) and cold 0.1 N acetic acid. The radioactivity remaining on the dried filters was determined.

Enzyme Assay

Cells were harvested and a crude membrane fraction was prepared as previously described (29). The membrane pellet was resuspended to a final concentration of 10 mg protein/ml in 20 mM Tris-Cl, pH 7.4, and 0.15 M NaCl (TBS). Membrane preparations were stored at 4°C for up to one week before use. Dolichol phosphate (80 μg/assay) was dried under N_2 and resuspended by sonication in 0.04% Triton X-100 in TBS. The assay mixture contained 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 M KCl, 20 mM Tris-Cl, 0.15 M NaCl, 103 μM UDP-N-acetyl-[³H]glucosamine (241 mCi/mmol), 0.5 mM dolichol phosphate, and 75 μg membrane protein in a final volume of 100 μl at pH 7.4. This mixture was incubated for 10 min at 34°C in a gyrotory water bath shaker (New Brunswick Scientific, New Brunswick, NJ), and the reaction was terminated by the addition of at least 20 vol of chloroform:methanol (2:1, vol/vol). The amount of incorporation of radioactivity into lipid was determined as previously described (29).

Inhibition of Enzyme Activity

Membranes (75 μg for wild-type cells and 10 μg for TM-resistant cells), TM (various amounts in 5 μl of 10 mM NaOH) and TBS in a total volume of 52 μl were preincubated at 25°C for 20 min before the addition of the remaining assay components (described above). After incubation for 10 min at 34°C, the reaction was terminated, the lipid fraction was extracted (29), and the amount of radioactivity in the lipid fraction was determined.

Silicic Acid Paper Chromatography

The ³H products were dried under N_2, resuspended in 50 μl chloroform:methanol (2:1, vol/vol), and chromatographed on Whatman SG-81 paper pretreated with EDTA (30) in a solvent system of chloroform:methanol:water (60:25:4, by vol) (31) for ~1.5 h. Each lane was cut into 5-mm slices which were then soaked in 0.8 ml of 1% SDS for 30 min at room temperature to elute the lipid. Radioactivity was measured by scintillation spectrometry.

DEAE-Cellulose Acetate Chromatography

The DEAE-cellulose acetate resin (Schleicher & Schuell, Inc., Keene, NH) was packed in a 1-×-10 cm column according to Rouser et al. (32) under air pressure. A small quantity of sea sand was layered above the resin. Labeled lipid samples were dried under N_2, resuspended in 1 ml chloroform:methanol:water (10:10:3, by vol), loaded onto the column, and eluted with an ammonium acetate step gradient in chloroform:methanol:water (10:10:3, by vol). After each use the column was washed and regenerated as directed (32).

Mild Acid Hydrolysis

Labeled lipids were dried under N_2, and resuspended in 50 μl of 0.1 N trifluoroacetic acid in 80% tetrahydrofuran. Samples were incubated at 50°C for 3.25 h. Hydrolysis was terminated by the addition of 4 ml of chloroform:methanol (2:1, vol/vol), and the samples were extracted as previously described (29).

Gel Filtration Chromatography

After mild acid hydrolysis and extraction, the water-soluble material was dried and then resuspended in a solution of sugar standards. This mixture was then applied to a 1-×-57 cm column of Biogel P-2 (-400 mesh) (Bio-Rad Laboratories, Richmond, CA) and eluted with 0.1 N Tris-Cl, pH 8.0, containing 0.1% NaN_3. Radioactivity of the fractions and the positions of the sugar standards were determined.

RESULTS

Resistance to TM In Vivo

After stepwise selection of a population of CHO cells resistant to 27 μg TM/ml, the cells were grown continuously in the presence of 27 μg TM/ml for several months. This TM-resistant population was 200-fold more resistant to the cytotoxic effect of TM than wild-type CHO cells. The dose-response curves in Fig. 1 show that wild-type cells have an LD_{50} for TM of 0.18 μg TM/ml, while the LD_{50} for the TM-resistant population is 35 μg TM/ml.

Morphology and Growth

During the selection procedure and subsequent maintenance of the culture in the drug, the TM-resistant cells were morphologically distinct from parental cells (Fig. 2). After removal of TM from the culture medium, the resistant cells retained their characteristic morphology for at least 4 mo. Growth curves indicated a generation time of 18 h for wild-type cells and 26 h for TM-resistant cells. The presence or absence of TM in the culture medium did not affect the generation time of the TM-resistant population. Even after 84 d of culture in the absence of TM, the generation time of the TM-resistant cells remained at 25 h. In the absence of TM the plating efficiencies were 84% and 62% for the wild-type and TM-resistant cells, respectively.
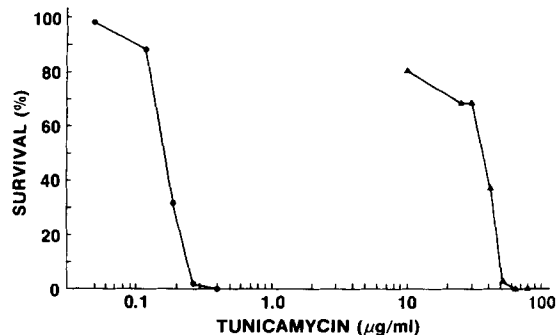
FIGURE 1 Effect of TM on wild-type and TM-resistant CHO cells. Wild-type and TM-resistant CHO cells were seeded at low density in MEM containing 5% FCS and various amounts of TM. After incubation at 34°C for 7 d, the cells were fixed with ethanol and stained with methylene blue. Only those colonies comparable in size to the colonies formed in the absence of TM were counted, although very small colonies were occasionally evident. The plating efficiencies were 84% and 62% for the wild-type and TM-resistant cells, respectively; the data were normalized to these values. (●) Wild-type cells. (▲) TM-resistant cells.

Mannose Incorporation In Vivo

The rate of asparagine-linked glycoprotein synthesis in wild-type cells in the absence and presence of TM (1 μg/ml) and in the TM-resistant cells in the presence of TM (27 μg/ml) was determined by measuring [^{3}H]mannose incorporation into TCA-precipitable, nonextractable macromolecules. Wild-type and TM-resistant cells exhibited comparable rates of glycoprotein synthesis in vivo (2.6 × 10^4 cpm/mg protein/h in wild-type cells and 2.1 × 10^4 cpm/mg protein/h in TM-resistant cells). In the presence of 1 μg TM/ml, wild-type cells incorporated label at a rate <4 × 10^3 cpm/mg protein/h. These results suggest that the cells are not resistant to the drug because they have a decreased rate of synthesis of asparagine-linked glycoproteins compared to wild-type cells, but rather that they have overcome tunicamycin inhibition by producing elevated levels of the N-acetylglucosamine-1-phosphate transferase.

N-Acetylglucosamine-1-Phosphate Transferase Activity In Vitro

The membranes of the TM-resistant cells (grown without drug for 16 d, see below) had 15-fold higher specific activity of N-acetylglucosamine-1-phosphate transferase than membranes from wild-type cells (279 pmol/mg/min and 18.6 pmol/mg/min, respectively). Incorporation of radioactivity into lipid increased linearly up to at least 150 μg protein per assay using membranes from wild-type cells; with membranes from TM-resistant cells, incorporation increased linearly only up to about 20 μg of protein (Fig. 3). The amount of membrane protein obtained from the two cell types was comparable (111 μg/10^6 wild-type cells and 169 μg/10^6 TM-resistant cells).

After growth for 3 mo (about 90 generations) in the absence of drug, the LD$_{50}$ of the TM-resistant cells was greater than that of wild-type cells (3.2 μg TM/ml vs. 0.18 μg TM/ml, respectively). The specific activity of the transferase was increased tenfold in the membranes from the TM-resistant cells.

FIGURE 2 Morphology of wild-type and TM-resistant CHO cells. Wild-type CHO cells were routinely maintained in MEM supplemented with 5% FCS, while the TM-resistant cells were maintained in MEM supplemented with 5% FCS and 27 μg TM/ml. The cells were seeded into 60-mm tissue culture dishes 2 d before photography. TM was not present in the culture medium of the TM-resistant cells during this period. (A) Wild-type cells. (B) TM-resistant cells. Bar, 50 μm. × 215.
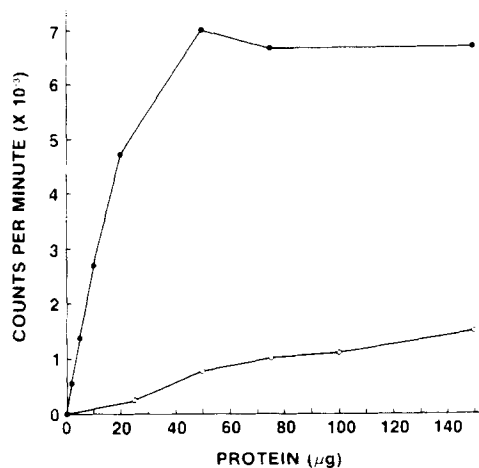

FIGURE 3 N-acetylglucosamine-1-phosphate transferase activity in membranes from wild-type and TM-resistant cells. Crude membranes from wild-type and TM-resistant cells were prepared and incubated with the reaction mixture as described in Materials and Methods. The UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-phosphate transferase activity was assayed by determining the radioactivity in the lipid fraction after a Folch extraction. Compensating amounts of membrane protein were added so that 150 μg protein was present during all extractions. (○) Wild-type cell membranes. (●) TM-resistant cell membranes.

Surprisingly, no enzymatic activity was observed in membranes from TM-resistant cells grown with TM up to the time of harvest. Membranes prepared from TM-resistant cells grown for 8 d without the drug had only 10% of the enzymatic activity measured in membranes of untreated wild-type cells, while membranes from TM-resistant cells grown without drug for 10–25 d showed maximum activity. The enzymatic activity of membranes from TM-resistant cells grown 14 days without drug was inhibited in vitro by TM, as was the activity of membranes from wild-type cells (Fig. 4). The EC_{50} of membranes from these TM-resistant cells was comparable to that of membranes from wild-type cells (0.03 μg TM/ml vs. 0.02 μg/ml, respectively). These results indicate that the transferase from the TM-resistant cells was sensitive to TM, and suggest that even after removal of TM from the cultures, some of the drug remained in the cell membrane for many generations.

Product Analysis

The products of the in vitro reaction using membranes of both wild-type and TM-resistant cells were N-acetylglucosaminylpyrophosphoryl-lipid and N,N'-diacetylchitobiosylpyrophosphoryl-lipid as analyzed by several chromatographic techniques. For all of these analyses, membranes were prepared from TM-resistant cells grown for 15–22 d without TM.

To determine the nature of the lipid moiety, silicic acid chromatography was used. In the solvent system employed, nonpolar lipids migrate with the solvent front while the migration of polar lipids is retarded. Silicic acid chromatography of [^{3}H]lipid fractions produced by membranes of both wild-type and TM-resistant cells migrated as two peaks, with R_f values of 0.35 and 0.25, and 0.32 and 0.25, respectively (Fig. 5A and B). In this system, [^{14}C]mannosylphosphoryldolichol ran with an R_f value of 0.54 (Fig. 5C). These results indicate that the products are not neutral lipids (which would have migrated with the solvent front), have a greater polarity than mannosylphosphoryldolichol, and are the same using either wild-type or TM-resistant cell membranes. The two peaks seen probably corresponded to N-acetylglucosaminylpyrophosphoryldolichol and N,N'-diacetylchitobiosylpyrophosphoryldolichol, respectively. This interpretation is in agreement with a previous report (33).

To further characterize the products, DEAE-cellulose acetate chromatography was employed to distinguish between lipids containing a monophosphoryl- and a pyrophosphoryl-linkage (33). Chromatography of the labeled products from membranes
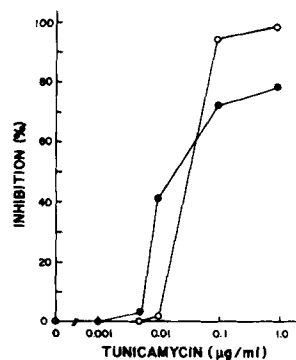

FIGURE 5 Product analysis by silicic acid chromatography. The in vitro products of the transferase reaction were chromatographed on Whatman SG-81 paper as described in Materials and Methods. Recoveries were 73% for the [^{3}H] label and 96% for the [^{14}C] label. O, origin. F, solvent front. (A) ^{3}H product from wild-type cell membranes. (B) ^{3}H product from TM-resistant cell membranes. (C) [^{14}C]mannosylphosphoryldolichol (standard).
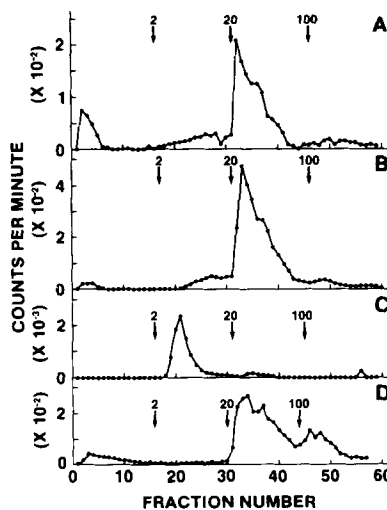
FIGURE 6 Product analysis by ion exchange chromatography. The in vitro transferase reaction products were chromatographed on a DEAE-cellulose acetate column by elution with an ammonium acetate step gradient of 2, 20, and 100 mM, as indicated in the figure. Equal fractions of ~2 ml were collected and analyzed for radioactivity. Recoveries ranged from 42–95%. (A) ^{3}H product from wild-type cell membranes. (B) ^{3}H product from TM-resistant cell membranes. (C) [^{3}H]glucosylphosphoryl-lipid (monophosphate standard). (D) [^{3}H]oligosaccharide-lipid (pyrophosphate standard).

of both wild-type and TM-resistant cells yielded a major peak eluting with 20 mM ammonium acetate (Fig. 6A and B). Authentic [^{3}H]glucosylphosphoryldolichol, a monophosphate standard, eluted at the 2 mM ammonium acetate step (Fig. 6C). [^{3}H]glucose-containing oligosaccharide-lipid, a pyrophosphate standard, eluted with 20 mM ammonium acetate (Fig. 6D). These results indicate that both products from membranes of both cell types are charged molecules which elute as pyrophosphoryl- rather than monophosphoryl-lipids.

Finally, the carbohydrate portion of the product was identified by gel filtration chromatography after mild acid hydrolysis of the glycosidic-phosphate bond. All of the label in the lipid fraction became water-soluble after treatment with mild acid. Typical gel filtration profiles of samples from membranes of wild-type and TM-resistant cells are shown in Fig. 7. The relative amounts of N-acetyl-[^{3}H]glucosamine and N,N'-diacetyl-[^{3}H]chitobiose varied with different membrane preparations; 25% or less of the radioactivity was found in the N,N'-diacetylchitobiose peak. Membranes of both cell types prepared and assayed on the same day yielded products with similar relative amounts of these carbohydrate moieties.

FIGURE 4 Inhibition of N-acetylglucosamine-1-phosphate transferase activity by tunicamycin. Crude membranes from wild-type and TM-resistant cells were prepared as described in Materials and Methods and preincubated at 25°C for 20 min with various concentrations of TM as indicated in the figure. The membranes were then incubated at 34°C for 10 min in the reaction mixture (see Materials and Methods) and the amount of radioactivity partitioning into the organic phase after extraction was determined. (●) Wild-type cell membranes. (○) TM-resistant cell membranes.
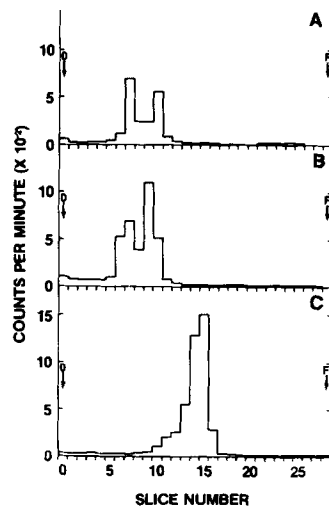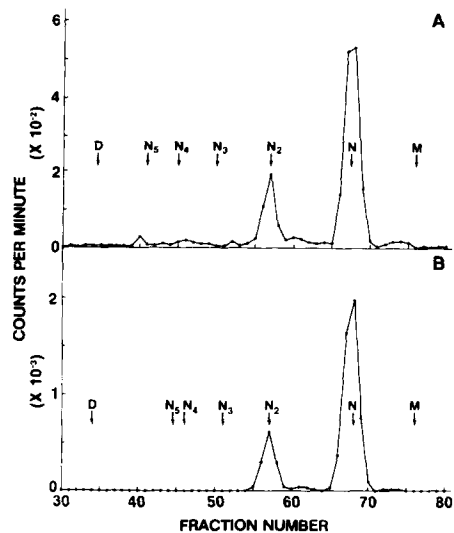
FIGURE 7 Product analysis by gel filtration chromatography. The sugar residues from the in vitro reaction products were chromatographed on a Bio Gel P-2 (-400 mesh) gel filtration column as described in Materials and Methods. Standards: M, mannose (internal volume marker); N, N-acetylglucosamine; N_x, linear N-acetylglucosamine polymers where x is the number of residues; D, dextran (void volume marker). Recoveries were 80-100%. (A) ^{3}H product from wild-type cell membranes; (B) ^{3}H product from TM-resistant cell membranes.

DISCUSSION

CHO cells resistant to TM have been isolated by employing a stepwise selection procedure with nonmutagenized cells similar to that described by Schimke et al. (34). These cells are capable of growing in medium containing 27 μg TM/ml, have an increased generation time compared to wild-type CHO cells and are morphologically distinct from the wild-type cells. Membranes prepared from the TM-resistant cells are able to synthesize N-acetylglucosaminylpyrophosphoryldolichol, and have a 15-fold higher specific activity of UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosaminylphosphate transferase than membranes from wild-type cells. This enzymatic activity remains sensitive to inhibition by TM.

Cellular resistance to cytotoxic agents, such as TM, may be due to a number of mechanisms, including overproduction of the target enzyme or protein, a decrease in the binding affinity for the cytotoxic agent, or an alteration in the transport apparatus for the compound. Various methotrexate-resistant murine and CHO cell lines have been isolated, and examples of each of these mechanisms have been described (34). Overproduction of the target enzyme appears to be a relatively common mechanism of resistance to tight-binding inhibitors; resistant "overproducer" cell types have been isolated using N-(phosphonacetyl)-L-aspartate (PALA) (4), pyrazofurin and 6-azauridine (7), hydroxyurea (35), β-aspartylhydroxamate (10), borrelidin (11), and methotrexate (6) as the selective agents.

Recently, TM-resistant CHO cell mutants have been isolated and described by two other laboratories. These mutants do not appear to have common characteristics with the TM-resistant CHO cells described in our paper. Two TM-resistant mutants isolated by Sudo and Onodera (36) exhibit resistance to a relatively low concentration of TM, and appear to have an altered permeability for TM. Kuwano et al. (37) have isolated CHO mutants resistant to both low and high concentrations of TM; the mutants resistant to a low dose of TM appear to be

transport mutants, while the mutants with a high level of resistance carry an additional mutation, which confers cross-resistance to 2-deoxy-D-glucose.

The mechanism of resistance to TM in the cells described here appears to be overproduction of the target enzyme, since we could demonstrate increased activity of the N-acetylglucosamine-1-phosphate transferase and sensitivity of this enzyme to TM. The possibility that the higher specific activity observed with membranes from the TM-resistant cells is merely a result of a lack of, or decrease in, substrate degradation during the assay has been considered. This is not the case, since (a) the concentration of UDP-N-acetylglucosamine used in the assay was saturating for wild-type enzyme, and (b) no degradation of the substrate was detected after the incubation period with either wild-type or TM-resistant cell membranes (data not shown). However, we have not ruled out the possibility that the dramatic increase seen in the specific activity of the transferase may reflect a higher affinity of the enzyme for its substrates. We are presently attempting to purify the UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-phosphate transferase from cloned derivatives of these TM-resistant cells.

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