Immunological Detection of Chinese Hamster Ovary Cells Expressing a Multidrug Resistance Phenotype

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ABSTRACT

A monoclonal antibody (IgG1) has been prepared that specifically detects Chinese hamster ovary cells expressing a multidrug-resistant phenotype. This antibody recognizes the membrane P-glycoprotein (Mr 170,000) associated with drug resistance as determined by enzyme-linked immunoabsorbent assay with purified P-glycoprotein and by Western blot analysis of cell extracts from drug-resistant and drug-sensitive cells. By immunofluorescence methods, the antibody also reacts strongly with viable and ether:ethanol-fixed resistant cells but does not react with the parent drug-sensitive cell line. Thus, this antibody can bind with live cells allowing discrimination by immunohistochemistry between drug-resistant and drug-sensitive Chinese hamster ovary cells.

INTRODUCTION

The treatment of human cancers with chemotherapy is often limited by development of resistance of the tumor cells to the cytotoxic agents. Drug resistance can either be preexisting or may develop under the selective pressure of chemotherapy. It is a clinical observation, that tumors resistant against one drug are often cross-resistant to various classes of other drugs.

The selection of drug-resistant cell lines exhibiting a similar phenotype of multidrug resistance has offered the opportunity to investigate the mechanism of this pleotropic drug resistance in vitro (1-6). A common denominator in pleitropic drug resistance seems to be a decreased intracellular accumulation of drug (7-10), which has led to the hypothesis that membrane alterations may be the cause of increased drug resistance. Among the best characterized cell lines expressing the pleitropic drug resistance phenotype are the colchicine-resistant mutants of CHO cells selected by Bech-Hansen et al. (1). The drug resistance phenotype has been found to be accompanied by increased expression of a plasma membrane glycoprotein with a molecular weight of 170,000, termed P-glycoprotein. This protein is either absent or present in trace amounts in plasma membranes of sensitive parent cells, and its expression in resistant cells correlates well with the degree of drug resistance (11-13).

A monoclonal antibody to P-glycoprotein of drug-resistant CHO cells has been prepared that specifically detects Chinese hamster ovary cells and show cross-reaction with drug-resistant and drug-sensitive cells. By immunohistochemistry this antibody can bind with live cells allowing discrimination by immunohistochemistry between drug-resistant and drug-sensitive Chinese hamster ovary cells.

MATERIALS AND METHODS

Materials. Antibodies were isotyped by ELISA using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). M-1500 polyclonal antibody was obtained from Koch-Light, Elk Grove, IL. Acrylamide, N,N'-methylene-bisacrylamide, and ammonium persulfate ultragrade reagents were purchased from LKB Instruments, Inc., Gaithersburg, MD, and protein standards for SDS gel electrophoresis were obtained from Bio-Rad Laboratories. Bacterial plasmid DNA was obtained from the plasmid DNA preparation kit (Boehringer Mannheim Biochemicals). Ricinus communis agglutinin I was obtained from Vector Laboratories, Burlingame, CA, and nitrocellulose filter paper (0.4-μm pore size) was from Schleicher and Schuell, Keane, NH. Immunol II 96-well microtiter dishes were from Dynatech, Alexandria, VA, and goat anti-mouse IgG peroxidase conjugate was from Cappel Laboratories, Cochranville, PA.

Cell Culture. The adenosine-, thymidine-, and glucose-requiring auxotroph AUXB₁, of CHO cells, hereafter called CHOAB, and its colchicine-resistant mutant CHC₅, hereafter called CHOC₅, were obtained from Dr. Ling of the Ontario Cancer Institute, Toronto, Canada. Cells were

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maintained as monolayer cultures in minimum essential medium (with ribonucleosides and deoxyribonucleosides) supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, pH 7.4, 0.2% sodium bicarbonate, and gentamicin (25 µg/ml; Irvine). The CHO5 line was maintained continuously in the presence of colchicine (1 mM) to prevent growth of revertants. Colchicine was removed at least one cell passage prior to experiments. Cell lines are routinely tested for Mycoplasma and found to be free of contamination.

**P-Glycoprotein Purification.** Isolation of plasma membranes of CHO cells and the purification of P-glycoprotein were performed with slight modifications according to the method of Riordan and Ling (19). Briefly, cells were ruptured with a Teflon-glass Potter-Elvehjem homogenizer in the presence of a hypotonic buffer (15 mM Tris (pH 8.0):1 mM MgCl₂:1 mM CaCl₂:1 mM PMSF). Homogenates were centrifuged at 800 x g to pellet nuclei and at 4,000 x g to pellet mitochondria. The microsomal pellet obtained by centrifugation at 40,000 x g was resuspended in homogenization buffer and applied to a discontinuous sucrose gradient (consisting of 60%, 45%, 31%, and 16% sucrose) and centrifuged for 18 h at 75,000 x g in an SW-27 swinging bucket rotor. Plasma membranes which concentrate at the 18:31% interface were drawn off and extracted with 0.5% N,N-dimethylsarcosine (Sigma) and 100 mM NaOH in 1 mM sodium bicarbonate, pH 7.4. The detergent extract was applied for final purification over a column of agarose-bound *R. communis* Agglutinin I. Protein was eluted from the column with 35 mM galactose (MCB Chemicals, Cincinnati, OH) and then dialyzed against PBS (8 mM sodium phosphate:2 mM potassium phosphate:137 mM sodium chloride:3 mM potassium chloride, pH 7.4) containing 1 mM PMSF.

**Immunizations and Production of Hybridomas.** Female BALB/c mice were given two i.p. injections at 1-wk intervals with 10⁵ viable CHOCS cells in 0.5 ml of serum-free growth medium. Three wk later, booster injections were given i.v. each day for 3 days prior to cell fusion, utilizing 40 µg of purified P-glycoprotein at Day 3 before fusion and 150 µg of plasma membrane extracts from CHO5 cells at Days 2 and 1 before fusion. Single cell suspensions of mouse spleenocytes were fused with NSI-1.Ag4-1 (NS-1) mouse myeloma cells as previously described. Details of cell fusion and hybridoma selection procedures have been published previously (20). Briefly, 50% polyethylene glycol was used as the fusing agent, and fused cells were plated at a concentration of 5 x 10⁵ cells/well in 96-well microtiter plates (Costar) in selection medium which consisted of RPMI-1640 medium supplemented with 20% fetal bovine serum (Hydclone Sterile Systems), 1% nonessential amino acids, 1 mM L-glutamine, 1 mM sodium pyruvate, Pen-strep (50 µg/ml), 0.1% hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine. Two wk after plating and hybridoma selection cultures were screened by ELISA for binding with the immunogen, CHO5 cells, cultures were rescreened for differential binding with membrane vesicles of parent and drug-resistant cells, and those showing a high CHO5:CHOAB binding ratio were taken for cloning. Cloning was by limiting dilution using nonirradiated BALB/c mouse T-cell feeder layers seeded at 5 x 10⁵ cells/well in 96-well microtiter dishes. Antibody products were obtained either as undiluted spent culture medium or as the IgG fraction of culture medium obtained from hybridoma cultures or purified MAbs. Plates were then washed 3 times with PBS and incubated for 3 h at room temperature with 150 µl of goat anti-mouse IgG peroxidase conjugate (Cappel) diluted 1:1500 with 1% BSA-PBS. Following incubation with anti-peroxidase conjugate, plates were washed 5 times with PBS and incubated for 10 min at room temperature with 150 µl of enzyme substrate (O-phenylenediamine:0.05% hydrogen peroxide, 2.5 mg/ml) prepared in citric acid buffer, pH 4.9 (50 mM citric acid monohydrate:0.1 M sodium phosphate dihydrate) and incubation was stopped by addition of 50 µl of 1 M sulfuric acid to each well. Plates were read in Dynatech MR-800 ELISA reader at a wavelength of 490 nm.

For ELISA using solubilized plasma membranes or purified P-glycoprotein as the target antigen, the antigen was diluted in 0.1 M borate buffer, pH 8.2 (10 µg/ml for membrane extracts and 2 µg/ml for purified P-glycoprotein), and absorbed to 96-well microtiter plates (Dynatech Immulon II) by overnight incubation at 4°C with 50 µl of these antigen solutions per well. Plates were washed twice in PBS and incubated for 1 h at 4°C with 1% BSA-PBS (150 µl/well) to block remaining sites. The remaining steps of the assay were the same as described above for fixed cell ELISA procedures.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting Procedures.** Proteins were resolved by SDS electrophoresis in polyacrylamide using the method of Fairbanks et al. (22), as modified by Debenham et al. (15). Briefly, this gel system consists of 5.6% polyacrylamide with a 3.75% bisacrylamide cross-linker, urea, and 1% SDS. No stacking gel is used, and sample buffer contains 2% SDS, 5% sucrose, 40 mM dithiothreitol, 1 mM EDTA, and 10 mM Tris, pH 8.0. Transfer of resolved proteins from gels to nitrocellulose filter paper was essentially as described by Towbin et al. (23). Protein transfer was performed for 16 h at room temperature at 150 mA using the Electroblot system of E-C Apparatus Corporation (St. Petersburg, FL). The electrode buffer was 20 mM Tris (pH 8.2):150 mM glycine:20% methanol. After transfer, additional protein binding sites on nitrocellulose were blocked by incubation of the paper for 2 h at 37°C in NEH buffer containing 3% bovine serum albumin, 0.25% gelatin, and 0.05% Triton X-100. The paper was then incubated overnight at 4°C with monoclonal antibody (diluted as indicated in figure legends) in a dilution buffer consisting of NEH buffer with 1% bovine serum albumin, 0.25% gelatin, and 0.05% Triton X-100, pH 7.4. After washing to remove unbound antibody (wash buffer is NEH containing 0.25% gelatin and 0.05% Triton X-100), the paper was incubated for 2 h at room temperature with a 1:2500 dilution of goat anti-mouse IgG-peroxidase in dilution buffer. After successive washes with wash buffer and substrate buffer (50 mM HEPES:150 mM NaCl, pH 7.4), the nitrocellulose blots were incubated with enzyme substrate for 30 min, which consisted of substrate buffer plus 20% methanol containing 4-chloro-1-naphthol at 1.0 µg/ml and 0.03% hydrogen peroxide. The reaction was stopped by rinsing with water. Efficiency of the electrophoretic transfer was monitored by Coomassie blue staining of gels following transfer and by Amido black staining of blots, and it was routinely greater than 90%.

**Indirect Immunofluorescence.** Tissue culture cells from the parent CHO5 line were plated in 8-well chamber slides in growth medium at 2 times 10⁵ cells/well and allowed to grow as a monolayer at 37°C for 24 h. Chambers were rinsed twice with PBS

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for 5 min and fixed with an ether:ethanol mixture (1:1) for 10 min. Following two additional washes with PBS, fixed cells were incubated with DEAE-cellulose-purified MAb 265/F4 (12.5 μg/ml) for 2 h at room temperature in a humidified chamber. Negative controls for each chamber slide included NS-1 myeloma spent culture medium, an unrelated mouse monoclonal antibody (anti-M, 24,000 estrogen-induced protein; Ref. 24), and phosphate buffer in place of the relevant MAb. Slides were washed twice in PBS and incubated for 2 h at room temperature with a 1:100 dilution of goat anti-mouse second antibody conjugated to fluorescein isothiocyanate (Cappel Laboratories; anti-mouse IgA, IgG, and IgM heavy and light chain specific). After final washing, slides were mounted with a medium consisting of 90% glycerol in PBS supplemented with p-phenylenediamine (1 mg/ml; Fischer Scientific, Fair Lawn, NJ). Using the phenylenediamine medium and storing slides in the dark at 4°C allowed viewing of the slides over the next 24 to 48 h without significant fading of fluorescence. Slides were viewed on a Leitz Dialux-20 fluorescence microscope equipped with a K480 filter to eliminate autofluorescence and photographed using Kodak Tri-X Pan film.

RESULTS

Demonstration of Drug Resistance. CHOCS and CHOAB cells were grown in the presence of four log doses of colchicine, ranging from 0.005 to 50 μg/ml. The 50%-inhibitory concentration values were determined for both cell lines as the dose at which 50% cell kill occurs compared with growth of untreated cells. The fraction of surviving cells (compared with untreated controls) remaining after 96-h incubation with the various doses of colchicine is shown in Chart 1. From these curves, 50%-inhibitory concentration of 17.5 μg/ml was obtained for the CHOC5 line and 0.15 μg/ml for the parent CHOAB line. Thus, a 117-fold resistance over parent cells was calculated for the CHOC5 cells used in our studies.

Selection of Monoclonal Antibodies Reactive with Drug-resistant CHO Cells. Mice were immunized with viable drug-resistant CHO cells as the primary immunogen. We know from the work of Ling and coworkers (1, 6, 9, 9, 12) that P-glycoprotein comprises a major component of the cell surface of drug-resistant CHO cells. The rationale behind the booster injection was to enrich the population of B-cells in the spleen specific for production of anti-P-glycoprotein and thus increase our chances of fusing and selecting the hybridomas of desired specificities.

Screening of the hybridomas was in three stages. The first level of screening was detection of binding by ELISA, with fixed drug-resistant CHO cells as the target antigen. This first level of screening selects all those cultures producing antibodies reactive with the immunogen (CHOCS) regardless of antigen specificity and eliminates all negative cultures. Positives were taken as those displaying ELISA binding 10-fold over background (i.e. ≥ 1.0 A, since background is in the range of 0.05 to 0.10 A). Of 158 wells containing hybridomas, 110 were positive on initial screening. The second level was to rescreen the positives for differential binding by ELISA with isolated plasma membrane extracts from resistant and parent CHO cells. An antibody against P-glycoprotein should give much higher binding to membranes of resistant cells than to membranes of parent drug-sensitive cells. Keeping in mind that hybridomas at this stage of screening are not clonal, we chose as our criterion for positivity a CHOC5:CHOAB binding ratio of ≥ 2.0. Upon rescreening, 12 cultures gave CHOC5:CHOAB binding ratios in the range of 2.0 to 6.0, and all 12 of these were subcloned by limiting dilution. Cloning microtiter dishes containing single colonies of cells were reassayed by ELISA first for binding to fixed CHOCS cells and second for differential binding with plasma membranes from CHOC5 and CHOAB cells. From this, one stable cloned cell line has been obtained which displays high binding to fixed drug-resistant cells and no detectable binding to the parent cell line. This hybridoma's cell line, which has been designated as 265/F4, secretes mouse IgG, has been shown to be clonal by repeated subcloning, and has produced MAb continuously for 1 yr. The binding by ELISA of the 265/F4 MAb (taken as spent culture medium from the cloned cell line) with membrane extracts of drug-resistant and -sensitive CHO cells is shown in Chart 2A. The 265/F4 MAb binds with high titer to membranes of drug-resistant cells, whereas no binding is detectable with membranes of the drug-sensitive parent cells. The polyclonal culture from which the 265/F4 MAb was isolated gave, by ELISA, a CHOC5:CHOAB binding ratio of about 6.0, whereas the cloned 265/F4 in Chart 2A shows no detectable binding to parent cells and thus an essentially infinite CHOC5:CHOAB binding ratio. This discrepancy is likely due to the fact that the polyclonal culture contained a mixture of antibodies to antigens, some common to both parent and resistant cells, and others specific for resistant cells. The high binding titer of the 265/F4 MAb to plates coated with lectin-purified P-glycoprotein (Chart 2B) suggests that this MAb is reactive specifically with the M, 170,000 P-glycoprotein of drug-resistant CHO cells. The third level of screening was by Western blot analysis to define the structure and specificity of antigen(s) recognized by the MAb (described below).

Antibody Detection of M, 170,000 P-Glycoprotein. Western blot analysis was used to determine the monospecificity and define the structure of the antigen(s) recognized by the 265/F4

Chart 1. Percentage of the surviving fraction of CHO cells after growth with increasing concentrations of colchicine. Parent CHOAB (O) and drug-resistant CHOC5 (∙) cell lines were plated in 25-cm² plastic T-flasks in regular growth medium (see "Materials and Methods") at a density of 2 × 10⁵ cells/flask. Two days after plating, growth medium was changed and supplemented with the concentrations of colchicine indicated. After 96 h of growth in colchicine, cells were harvested with trypsin:EDTA and counted, and the percentage of surviving cells was determined for each dose of drug. Each point represents the average of duplicate treatment flasks.
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Chart 2. ELISA of 265/F4 binding to CHO membrane extracts and lectin-purified P-glycoprotein. In A, membranes were isolated from CHOAB (O) and from CHOC5-resistant cells (•), and detergent extracts at a concentration of 10 µg of protein per ml from each were bound to plastic wells of microtiter dishes. Antibody binding was assessed by ELISA as described in "Materials and Methods." Spent hybridoma culture medium was used as a source of antibody and was diluted as indicated in 1% BSA:PBS. In B, microtiter plates were coated with lectin-purified P-glycoprotein from CHOC5 drug-resistant cells at a concentration of 2 µg/ml. Binding of 265/F4 to P-glycoprotein-coated plates (•) was by ELISA as above. ELISA binding to P-glycoprotein-coated plates was also determined with an unrelated mouse monoclonal IgG1 (anti-M, 24,000; Ref. 25) and NS-1 spent culture medium. Both negative controls gave equivalent low binding (O).

Fig. 1. Cell fractionation of drug-resistant CHOC5 (Lanes 5 to 8) and drug-sensitive CHOAB cells (Lanes 1 to 4), and their subsequent Western blot analysis. In the top, Coomassie-stained SDS-polyacrylamide electrophoresis gels of cell fractions, 100 µg of total cell homogenates (Lanes 4 and 5), 100 µg of microsomal 40,000 x g pellet (Lanes 3 and 7), 100 µg of plasma membranes (Lanes 2 and 6), and 20 µg of R. communis lectin affinity eluate (as described in "Materials and Methods") from plasma membranes (Lanes 1 and 7) were layered onto respective gel lanes. Gels were stained with Coomassie-blue as described in "Materials and Methods," and molecular weight standards were from Bio-Rad: myosin (M, 200,000); b-galactosidase (M, 116,500); phosphorylase B (M, 92,500); bovine serum albumin (M, 66,200); ovalbumin (M, 45,000). In the bottom, Western blot analysis of the same cell fractions from above electrophoresed on a parallel SDS-polyacrylamide gel, transferred to nitrocellulose filter paper, and reacted with monoclonal antibody 265/F4 as described in "Materials and Methods" is seen.

MAb. Various cell fractions, including whole cell homogenates, microsomes, isolated plasma membranes, and lectin-purified membrane glycoproteins, were resolved and analyzed by SDS:gel electrophoresis. Coomassie-stained gels of each cell fraction are shown in the top panel of Fig. 1 (Lanes 5 to 8). For comparison, Coomassie-stained SDS:gels of the same fractions isolated from CHOAB parent cells are also shown in the top panel of Fig. 1 (Lanes 1 to 4). A predominant protein band at M, 170,000 is visible in the plasma membrane fraction of drug-resistant cells (Lane 6) which is not detectable in membranes isolated from the parent cell line (Lane 2). Moreover, the Ricinus lectin eluate from membranes of resistant cells contains a single M, 170,000 protein band (Lane 5) which is also not detectable in drug-sensitive parent cells (Lane 1).

These same cell fractions were resolved on parallel SDS:gels, transferred to nitrocellulose filter paper, and then reacted with the 265/F4 antibody as described in "Materials and Methods." As shown in the bottom panel of Fig. 1 (Lanes 5 to 8), immunoreactivity was detected with a single M, 170,000 protein band in all the CHOC5 cell fractions, including the crude cell homogenate (Lane 8) and the lectin-purified P-glycoprotein (Lane 5). Some smaller molecular weight bands were also reactive in the lectin eluate (Lane 5) as well as isolated membrane fractions (Lane 6), but these most likely represent degradation products since only a single M, 170,000 reactive band is seen in the crude cell homogenate. No cross-reaction was detected with any other bands in these complex mixtures of proteins. The high sensitivity of this antibody is illustrated by the fact that M, 170,000 P-glycoprotein is detectable in crude homogenates of CHOC5 (Lane 8), yet a M, 170,000 band is not readily visible in the stained gels of this same crude fraction. By contrast to the results obtained with CHOC5 cells, no immunoreactivity was detected in any of the cell fractions isolated from parent CHOAB cells as shown in Lanes 1 to 4 of the bottom panel in Fig. 1. Thus, the 265/F4 MAb appears to be highly specific for a M,
170,000 protein of drug-resistant cells and shows little or no cross-reaction with the drug-sensitive parent cells.

**Immunohistochemical Detection of Drug-Resistant Cells.** Use of the 265/F4 antibody for immunohistochemical detection of CHO cells was investigated utilizing an immunoperoxidase procedure with Bouin’s fixed cells embedded in paraffin and by indirect immunofluorescence as described in “Materials and Methods.” No peroxidase staining was observed with fixed/embedded cells (either parent or resistant), indicating that antigenic determinants do not withstand fixation and/or subsequent processing for paraffin embedding. However, strong immunofluorescent staining of CHO5 cells, lightly fixed in ethanol/ether, was observed. As shown in Fig. 2a, intense membrane immunofluorescent staining was observed in the resistant CHO5 cells. Staining was heterogeneous in the sense that fluorescence of some cells was greater than that of others. By contrast, no specific membrane immunofluorescence was observed with parent CHOAB cells as illustrated in Fig. 2b. Exposure time for photography was the same in Fig. 2a and b. The same fields of view photographed for fluorescent staining were also photographed under white light as shown in Fig. 2c and d, respectively. The photomicrographs illustrate that immunofluorescent membrane staining observed with CHOCS cells was with nearly every cell, while parent CHOAB cells uniformly lacked specific immunofluorescence. Viable unfixed cells were also tested for immunofluorescent staining with 265/F4. We again observed specific membrane staining only in the drug-resistant CHO5 cells (data not shown).

**DISCUSSION**

The mechanisms of multidrug resistance in vitro are not completely understood. The degree of cross-resistance and the drugs involved in the process vary among different cell lines, which indicates a complex mechanism (2). However, appearance of a single membrane alteration in the form of increased expression of high molecular weight glycoproteins such as P-glycoprotein suggests the possibility for a common mechanism as the result of a single gene alteration. Utilizing polyclonal antisera to P-cells (data not shown).

...the role and function of P-glycoprotein in the mechanism of the multidrug resistance phenotype. Since the MAb is capable of detecting antigen on the surface of live cells, it will be of value in isolating drug-sensitive and -resistant cells from mixed populations by fluorescence-activated cell sorting techniques. It may also be useful as a probe to explore the possibility that P-glycoprotein may display microheterogeneity and qualitative differences in drug-resistant cells compared with -sensitive cells and in immunofluorescence purification of P-glycoprotein to use as immunogen to generate further MAbs against different epitopes and cross-reactive with human antigen.

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