Low External pH and Osmotic Shock Increase the Expression of Human MDR Protein†

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ABSTRACT: We have studied the effects of extracellular pH (pH_o) and osmotic strength on the expression of the human multidrug resistance (MDR) protein. Both lowered pH_o and hypertonic shock increase the level of hu MDR protein 5–10-fold in membranes isolated from the human colon carcinoma cell lines SW620 and HCT15 and the human kidney carcinoma line SKRC-39. Increased protein expression is dependent on the duration of acid or osmotic shock and is reversed within several days when normal growth conditions are restored. Quantitative northern blot analysis with a hu MDR 1 specific probe reveals increased MDR mRNA in the acid and hypertonically shocked cells. Interestingly, we find a greater increase in mRNA levels for *hypotonically* shocked colon cells, *without* an apparent increase in protein levels. Overexpressing cells are found to retain less [^{3}H]vinblastine relative to cells cultured under normal conditions, and they are resistant to the cytotoxic effects of doxorubicin, vinblastine, and colchicine, but not methotrexate. This resistance appears to be reversed by treatment with verapamil. In contrast, SW620 cells previously induced to overexpress MDR protein via the administration of differentiation agents [Mickley *et al.* (1989) *J. Biol. Chem. 264,* 18031–18040] did not exhibit decreased retention of [^{3}H]vinblastine; thus low-pH_o-induced overexpression of MDR protein in these cells may provide additional factors that promote the full expression of the MDR phenotype. These data may help to explain why many solid tumors (e.g., of colon and kidney origin) develop MDR prior to chemotherapy, since they usually grow under similar acidic conditions. These data also support the contention that MDR protein may play a role in intracellular pH and volume homeostasis.

Overexpression of the multidrug resistance (MDR[1]) protein, or P-glycoprotein, confers the MDR phenotype to tumor cells (Endicott & Ling, 1989; Gottesman & Pastan, 1993). Overexpression is typically induced by selecting tumor cells on increasing concentrations of certain chemotherapeutic drugs. MDR cells are resistant not only to the selecting agent but also to other antineoplastics as well, including (in general) anthracyclines, vinca alkaloids, and colchicine, and they are also resistant to nonchemotherapeutic compounds like gramicidin D and tween 80. Resistance to the chemotherapeutics is due in part to decreased intracellular retention of the drugs over time. Because of the homology between MDR protein and several bacterial transporters (Gros *et al.*, 1986; Chen *et al.*, 1986) as well as photolabeling studies with chemotherapeutic drug analogues (Safa, 1988; Cornwell *et al.*, 1986), it has been proposed that MDR protein lowers intracellular levels of chemotherapeutics by performing active drug transport in the outward direction (i.e., active drug efflux). However, it is not understood how a single transport protein could actively efflux the dozens of structurally dissimilar compounds to which MDR cells are resistant.

An alternative or perhaps complementary [see Gill *et al.* (1992)] hypothesis for MDR is that the MDR protein elevates intracellular pH (pH_i) and/or lowers plasma membrane electrical potential ($\Delta\Psi$) such that lipophilic weakly basic and/or cationic drugs or drugs that bind to intracellular targets in a highly pH dependent manner are less likely to be retained over time [Roepe *et al.*, 1993; see also Beck *et al.* (1983) and Siegfried *et al.* (1985)]. Consistent with this hypothesis is the frequent observation of alkaline pH_i in a variety of MDR cells overexpressing MDR protein (Keizer & Joenje, 1989; Boscoboinik *et al.*, 1990; Thiebaut *et al.*, 1990; Roepe, 1992; Roepe *et al.*, 1993; Luz *et al.*, 1994) and altered translocation of chloride in cells transfected with the *MDR 1* gene relative to controls (Valverde *et al.*, 1992; Gill *et al.*, 1992), since the distribution and transport of chloride impacts greatly on $\Delta\Psi$ and pH_i. Parenthetically, chloride translocation by MDR protein in some cells is apparently stimulated by hypotonic shock (Valverde *et al.*, 1992; Gill *et al.*, 1992), which leads to cell swelling and changes in pH_i.

Furthermore, it has recently been demonstrated that elevated pH_i is linearly related to the relative steady state level of anthracycline efflux for a series of four MDR cell lines expressing different levels of MDR protein when the cells are preloaded to the same total intracellular level of drug, and that the rate of drug efflux from the different cells is identical when the cells are preloaded to the same level of exchangeable intracellular drug (Roepe, 1992). These data suggest that, at least in some cells, MDR protein promotes decreased retention (binding) of drug without actually performing active drug transport [see also Beck *et al.* (1983)]

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[1] Abbreviations: MDR, multidrug resistance; pH_i, intracellular pH; $\Delta\Psi$, plasma membrane electrical potential; Δ pH, pH gradient across the plasma membrane; $\Delta\mu_{H^+}$, plasma membrane electrochemical potential ($\Delta\mu_{H^+} = \Delta\Psi + \Delta$ pH); V_i, intracellular volume; RVD, regulatory volume decrease; pH_o, extracellular pH; HPLC, high-pressure liquid chromatography; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; SNARF, carboxy-seminaphthorhodafluor-1; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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and that in some cases decreased retention may be due to altered pH_i homeostasis. We have also recently shown that $\Delta\Psi$ decreases as MDR protein expression increases in the same series of MDR cell lines (Roepe *et al.*, 1993) and that this loss of electrical potential energy is compensated for by the gain in chemical potential associated with increased ΔpH. Thus, viewing pH_i perturbations in a bioenergetic context may be helpful in some cases. Decreased $\Delta\Psi$ could explain why MDR cells are frequently resistant to lipophilic cations, detergents, and ionophores whose biological activity is dependent to some extent on the character and magnitude of $\Delta\mu_{H^+}$. In any case, it is also apparent from these studies that pH_i homeostasis is dramatically altered in some MDR cells, and that some MDR cells exhibit rather dramatic overexpression of cation (Na^+/H^+) and anion ($Cl^-/^-HCO_3$) exchangers, as well as subtle changes in intracellular volume (V_i) (Roepe *et al.*, 1993; Luz *et al.*, 1994).

It is well appreciated that some ion transporters involved in pH_i homeostasis are also involved in the regulation of V_i (Hoffman & Simonsen, 1989; Moolenaar, 1986; Grinstein *et al.*, 1983, 1985). For example, when Ehrlich ascites tumor cells are hypotonically shocked, a "regulatory volume decrease" (RVD) response is initiated, wherein K^+ and Cl^- currents are increased, and Na^+ current is decreased. Compensatory H^+ uptake to electrically balance K^+ loss during RVD leads to net intracellular acidification in these cells, since it apparently outpaces $Cl^-/^-HCO_3$ exchange and swamps intracellular buffering capacity [see Hoffman and Simonsen (1989) and references within].

One prediction of the hypothesis that MDR protein has a pH_i and/or V_i regulatory function is that pH_o or pH_i alterations might modify the relative activity of MDR protein, or perhaps even induce/repress its expression, as is the case for the other genes involved in pH_i regulation (Auger *et al.*, 1989; Slonczewski, 1992). This would have important implications in light of the fact that most solid tumors grow within an acidic environment (Jain *et al.*, 1984; Rotin *et al.*, 1989; Wike-Hooley *et al.*, 1984) and may exhibit MDR prior to exposure to chemotherapeutics. Tissue culture conditions that transiently lower pH_i would include lower pH_o and perhaps RVD.

We have investigated these possibilities by culturing human kidney carcinoma and human colon carcinoma cell lines in various CO_2 environments and under hypotonic and hypertonic conditions.

Since solid tumors in general grow faster than vasculature and lymph develop around them, the data in this paper imply that some tumor cells might develop MDR as fluid surrounding the tumor is acidified. They also indirectly support the hypothesis that MDR protein plays a role in pH_i homeostasis, intracellular volume regulation, and/or the maintenance of plasma membrane electrochemical potential, and they highlight the importance of careful tissue culture technique in quantitative examination of MDR phenomena in some cell lines.

MATERIALS AND METHODS

Materials. [^{3}H]Vinblastine sulfate (16 Ci/mmol) and the ECL western blotting detection kit were purchased from Amersham International. Purity was >95% as assayed by HPLC with a Hypersil MOS column. The monoclonal antibody C219 is the product of Centocor Incorporated, and horseradish peroxidase conjugated anti-mouse IgG was from Sigma. Adriamycin was obtained as a 2 mg/mL solution in saline from Adria Laboratories, and colchicine was purchased from Sigma. Vinblastine sulfate was a kind gift of Lilly Research Laboratories, and methotrexate was obtained as a 25 mg/mL solution in saline from Lederle Laboratories. 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), carboxy-seminaphthorhodafluor-1 (SNARF), nigericin, and valinomycin were from Molecular Probes (Eugene, OR). All other chemicals were reagent grade or better and purchased from commercial sources.

Tissue Culture. The human colon carcinoma lines SW620 (obtained from ATCC) and HCT15 (kindly provided by the laboratory of Dr. Joseph R. Bertino, Sloan-Kettering Institute) were maintained in RPMI-1640 media supplemented with 10% fetal calf serum and 100 units/mL penicillin and 100 μ/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. Care was taken to ensure that stock cultures were never grown to complete confluency, since in our hands this is associated with a mild change in medium pH (pH_o). SKRC-39 cells were originally established from a renal biopsy (Ebert *et al.*, 1990) and were a kind gift of Dr. James P. O'Brien, Sloan-Kettering Institute. They were grown in MEM medium with 10% fetal calf serum and additional amino acids and antibiotics as above. To acid shock cells, they were grown in an elevated CO_2 atmosphere in RPMI-1640 medium initially containing 27 mM $^-HCO_3$. At 10% CO_2, medium pH after 24 h was 6.8 for a culture seeded at 1×10^6 cells/100 cm^2. After 3 days of growth at 10% CO_2, the by now yellowish medium exhibited a pH of 6.5–6.6, at which time the cells were usually fed fresh medium preequilibrated in a 10% CO_2 atmosphere. Thus, note that pH_o is dependent to some extent on cell density as well as percent CO_2. Notably, since growth rates under the different conditions were nearly identical (see Results), these mildly acidic culture conditions do not deleteriously affect the integrity of the medium such that cells are deprived of essential nutrients or factors. Although some mild variability in medium pH_o can be observed for different cultures grown under a similar percent CO_2, in our initial experiments we did not wish to include the added complexity of additional artificial buffers. Thus, medium pH_o was checked repeatedly, and all efforts were made to standardize these procedures by preequilibrating media, by plating exactly the same number of cells/dish, and by growing cultures for similar periods of time.

Hyperosmotic shock was performed by addition of NaCl to normal RPMI or MEM culture medium (additional 25–75 mM, see Results). The volume of the culture medium was checked daily to ensure that osmolarity was not further altered by evaporation. Hypotonic shock was performed by addition of H_2O (or NaCl-free medium) to the normal medium (additional 1–5 mL to 10 mL of medium; levels of fetal calf serum, penicillin, streptomycin, and amino acids were kept constant by supplementing the diluted medium with stock solutions of the other components). When the medium was made more than 10% hypotonic, water or NaCl-free medium was added at the rate of 10%/h so that the cells had time to adjust; similarly, salt was added gradually to produce some hypertonic conditions. Under extreme osmotic stress, some cells died within a 12–24-h exposure; these were removed by washing and addition of fresh medium. All subsequent analyses were performed with viable cells, as assayed by dye exclusion and microscopy.

Cell Survivability. For growth inhibition assays, cells were subcultured in 96 well plates and incubated overnight at 5% CO_2. Drug at various concentrations were added the following day, and cells were grown for 3 days more at 5% CO_2. Cell growth was assayed by crystal violet staining as described (Prochaska & Santamaria, 1988). We also verified that staining was a linear function of both cell number and total

cell-associated protein (S. Basu and P.D.R., unpublished) for these cell lines grown under the different conditions.

Since overexpression of MDR protein in these cell lines is reversed upon continued growth at 5% CO_2, we also assayed resistance by a cytotoxicity assay involving shorter exposure to drug. Cells were harvested by trypsinization and resuspended in RPMI-1640 medium preequilibrated to 37 °C and 5% CO_2. The pH_o of the medium was checked before and after the experiment. Then 10^5 cells were aliquoted into eppendorf tubes harboring increasing concentrations of chemotherapeutic. Cells were incubated with drug at 37 °C, 5% CO_2, while being gently rotated for 3 h. After exposure to drug, cells were washed twice in preequilibrated medium and resuspended in 1 mL of medium, and 200-μL aliquots were added to individual wells of a 96-well plate. Cells were grown at 37 °C, 5% CO_2, for 4 days and then stained with crystal violet as above. In each experiment, cells exposed to a given concentration of drug were plated in triplicate or quadruplicate, and results from three experiments were averaged to produce the plots presented in Results. In these experiments we wished to keep pH_o constant for both cell types during exposure to drug (different pH_o would affect relative uptake of the drugs). Thus, preequilibrated medium was used, and pH_o was checked repeatedly.

Northern Blotting. RNA was isolated by the method of Chomczynski and Sacchi (1987), and northern blots were performed as described (Roepe *et al.*, 1993). We used 0.7-kbp *Nde*I/*Hin*dIII and 1.9-kbp *Bam*HI fragments from hu MDR 1 cDNA (kindly provided by P. Borst) and hu NHE 1 cDNA (kindly provided by J. Pousséygur), respectively, to probe for hu MDR 1 and NHE 1. We used 1.1-kbp *Eco*RI and 0.8-kbp *Pst*I fragments from hu β-actin cDNA and acidic ribosomal phosphoprotein (rPO) cDNA, respectively (both kindly provided by K. Scotto), to probe for β-actin and rPO. Probes were labeled by the random priming method. Blots were visualized by autoradiography (exposure times varied from 4 to 6 h for β-actin and rPO to 16 to 24 h for NHE and MDR), and levels of mRNA were quantitated by imaging β radiation with a betascope 603 blot analyzer (Betagen). The intensities of bands of interest were normalized relative to background emission from the blot. Since some levels of relative overexpression were low (see Results), quantitation of all blots was confirmed by performing at least three separate northerns using at least three separate RNA preparations isolated from at least three separate cell cultures.

Western Blotting. Cells were harvested after growth at various percent CO_2, snap-frozen with dry ice/ethanol, and stored at −80 °C. Membrane fractions were prepared by resuspending cells in 10 mM Tris/1 mM MgCl/1 mM PMSF/0.5 mM DTT (TMPD) buffer, pH 8.0 at 4 °C, homogenizing with a Dounce homogenizer (50–75 strokes), and centrifuging the homogenate over a 45% sucrose cushion made in the same buffer. The interfacial layer was collected, washed three times in TMPD to remove sucrose, and stored at −80 °C. Protein determination of uniform suspensions was by an amido black assay with BSA used as a standard. Membrane protein (20 μg) was solubilized in cracking buffer (10 mM, Tris, pH 8.0/10% glycerol/0.01% bromophenol blue/1 mM DTT) at 37 °C, and proteins were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis. Protein was blotted to nitrocellulose (Bio-Rad) at 40-mA constant current, and western blots using the monoclonal C219 were performed as described (Luz *et al.*, 1994). Antibody was detected by the enhanced chemiluminescence (ECL) method (Amersham); exposure times varied between 2 and 5 min. Relative expression of MDR protein was calculated after densitometry of the relevant bands using a Stratagene Stratascan 7000 densitometer; band intensities were normalized relative to background emission from the blot.

Drug Retention. Cells were subcultured in six-well plates at 5×10^5 cells/well and then incubated overnight at 5% CO_2 before the assay was performed. Each well was incubated with 0.5 μM [^{3}H]vinblastine (200 mCi/mM) at 5% CO_2. The wells were then washed with cold medium, to remove uninternalized vinblastine, and incubated with warm medium with intermittent gentle swirling. Radioactivity in the cells, incubation medium, and wash medium was assayed by liquid scintillation spectrometry. Total cell-associated protein was quantitated by an amido black assay. We verified that the average protein content per cell was similar for the cells grown for 3–4 days at lower pH_o vs control cells grown under normal conditions. Thus, although volume and protein content per cell likely changes within the first several hours of exposure to these extrema, by 3–4 days' growth under the different conditions the cells have adapted (see also Results).

Measurement of Intracellular pH and Na^+/H^+ Antiport Activity. Intracellular pH was calculated as described (Roepe, 1992; Roepe *et al.*, 1993; Luz *et al.*, 1994) by measuring the ratio of fluorescence excitation at 439 and 505 nm while monitoring emission at 535 nm for cells in continuously mixed suspension (1.5×10^5 cells/mL) or grown on coverslips preloaded with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). To load cells with dye, they were incubated in 5 μM acetomethoxy BCECF (BCECF-AM) for 30 min at 37 °C. The cells were then washed in Hank's balanced salts (HBSS) or HEPES balanced salts (HBS) with glucose and rapidly injected into a continuously mixed cuvette before fluorescence spectra were obtained. A standard curve was generated by the K^+/nigericin method. Leak of dye was not different for different cells and was virtually nil during the time course of the measurement (<3 min/sample). Standard curves were generated for each cell preparation each time a measurement was made.

In some cases, pH_i was also calculated using carboxy-seminaphthorhodafluor-1 (SNARF), which has two distinct pH sensitive emission maxima. Emission at 587 and 635 nm was ratioed, while excitation was constant at 535 nm. In virtually all other respects, the procedure was the same as for BCECF.

In addition, pH_i was assessed for individual cells by single-cell photometry methods as described (Luz *et al.*, 1994). BCECF vs pH_i standardization curves were performed for each coverslip by the K^+/nigericin method using buffer preequilibrated to the desired pH_o. Each point in the curve was the average of the signal from at least 20 different cells, and linear curves were obtained for $6.6 \leq pH_i \leq 7.0$ and $7.0 \leq pH_i \leq 7.9$. pH_i was calculated for at least 12 individual cells and then averaged.

To measure Na^+/H^+ antiport, cells were first loaded with BCECF as above, pH_i was lowered in Na^+ deficient balanced salts as described [see Roepe *et al.* (1993) for a more complete discussion], and Na^+ was added to induce recovery. Recovery was sensitive to amiloride [see Roepe *et al.* (1993)]. These experiments could be performed either with cell suspensions or with single cells grown on glass coverslips.

RESULTS

Decreased pH_o Lowers pH_i for Colon Carcinoma Cells. The colon carcinoma cell lines SW620 and HCT15 are easily grown under a variety of cell culture conditions and can be

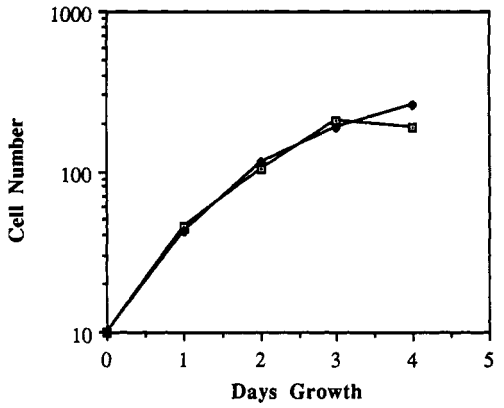

FIGURE 1: Growth of SW620 colon carcinoma cells in standard RPMI medium in an atmosphere of either 5% CO_2 (solid tilted squares) or 10% CO_2 (open squares). Cells were plated in six-well plates, and individual wells were harvested at different times and the cells counted with a Coulter counter. Each point represents the average of three experiments. Note that the rates of growth are very similar under the two sets of conditions, whereas for many other cell types (including NIH 3T3 fibroblasts or DC-3F chinese hamster lung fibroblasts, data not shown) growth is slower in an environment of 10% CO_2. Similar growth rates at the two percent CO_2 are also seen for HCT 15 colon and SKRC-39 kidney cells (not shown).

grown in standard RPMI-1640 medium and a 10% CO_2 environment with little effect on growth rate, relative to growth at 5% CO_2 (Figure 1). Compared to many other eukaryotic cell lines they are thus better able to respond to external pH (pH_o) perturbations. An atmosphere of 10% CO_2 quickly lowers the pH of standard RPMI-1640 medium to about 6.8 (not shown). As the colon carcinoma lines grow in this environment, pH_o is further reduced over a period of several days to about 6.6–6.5 as the culture approaches confluency (not shown). In contrast, cultures grown at 5% CO_2 develop a pH_o of about 7.0 at confluency, from a starting point of 7.4–7.3. These trends in pH_o are highly reproducible, assuming cells are treated identically each time. We thus did not use additional buffers (such as HEPES) in these experiments, although in the future it may prove worthwhile to investigate their utility. Greater than 95% of these cells are viable after 5 days' growth at 10% CO_2, as assayed by dye exclusion.

It is likely, therefore, that SW620 and HCT15 cells adapt to lowered extracellular pH (pH_o) by acquiring mechanisms for alleviating an increased intracellular acid burden (i.e., elevating pH_i). Indeed, in either mass population or single-cell measurements (see Methods) SW620 cells grown at 5% CO_2 are found to exhibit pH_i values of approximately 6.8 when pH_o is transiently lowered to 6.5, whereas cells grown at 10% CO_2 for 5–6 days exhibit pH_i values of approximately 0.15–0.3 pH units higher, respectively, at a similar pH_o (Figure 2A, compare open and closed squares). Similar behavior is seen for HCT15 (Figure 2B) although these cells exhibit slightly higher pH_i values relative to similarly treated SW620 cells.

In addition, pH_i is found to be more alkaline for cells grown in an atmosphere of 10% CO_2 relative to cells grown at 5% CO_2 when both sets of cells are incubated at 5% CO_2 for 12 h (cf. Figure 2, diamonds) before pH_i is determined. That is, the acid-shocked cells "overshoot" pH_i when they are returned to normal culture conditions. This overshoot is gradually reversed after about 2–3 days' additional growth at 5% CO_2 (not shown).

Acid Shock Increases MDR Protein Levels. Since these cells express measurable but low levels of MDR protein and since there is some indication that increased MDR protein

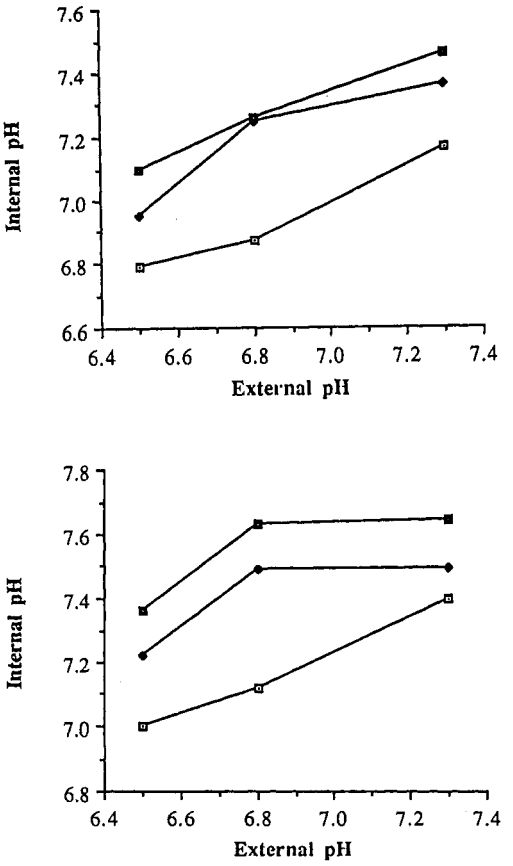

FIGURE 2: Internal pH (pH_i) for SW620 (A, top) or HCT15 (B, bottom) cells at several pH_o. Cells were either grown continuously at 5% CO_2 ("control"; open squares), grown for 5 days at 10% CO_2 and then for 12 h at 5% CO_2 (5 × 10%, 1 × 5%; solid tilted squares), or grown for 6 days at 10% CO_2 (6 × 10%; solid squares) before loading with BCECF (see Methods). Leak of dye, which was miniscule during the time course of the measurement, was not any different for the different cells. Note that pH_i is higher at all values of pH_o for the acid-shocked cells, relative to the cells grown continuously at 5% CO_2. Values shown are the averages of multiple determinations using mass cell populations (SE < 2% for pH_i; pH_o is ± 0.03 units); these data are in agreement with other experiments that measured pH_i via single-cell photometry (data not shown).

expression may correlate with intracellular alkalinization for some cells (Keizer & Joenje, 1989; Roepe, 1992), we measured relative expression of hu MDR protein. Figure 3 summarizes three different western blot analyses of membranes isolated from SW620 (lanes 1, 2), HCT-15 (lanes 3, 4), and SKRC-39 (lanes 5, 6) cells grown in an environment of 5% (left side) or 10% (right side) CO_2 for 3–5 days. Quite interestingly, by densitometry it is determined that at least 10-fold more MDR protein per milligram of total membrane protein is present for the SW620 colon cells grown in an acid environment for about 5 days, relative to identical cells grown at 5% CO_2. Similarly, an approximately 4–5-fold increase in MDR protein content is seen for the HCT15 colon and SKRC-39 kidney cells grown at 10% CO_2 for 3 and 5 days, respectively, relative to cells grown at 5% CO_2 (Figure 3). Overexpression in the SW620 colon cells is reminiscent of increased MDR protein expression observed previously for the same cell line upon exposure to the differentiation agents DMSO or sodium butyrate (Mickley *et al.*, 1989); however, note that the peroxidase-enhanced detection method we use is more sensitive than the ^{125}I-protein A method used previously. Thus, although it is difficult to compare absolute levels of protein detected via the two methods, fold overexpression seen in Figure 3 is likely less than the maximal fold overexpression seen previously upon 6

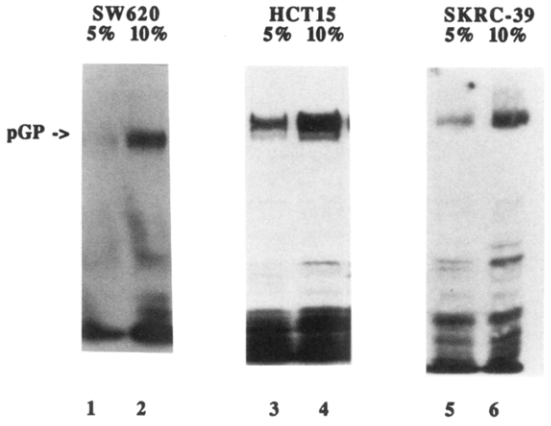

FIGURE 3: Western blot analysis of MDR protein expression in membranes isolated from SW620 (left), HCT15 (middle), and SKRC-39 (right) cells grown either at 5% CO_2 (left side, each comparison) or for 3–5 days in an atmosphere of 10% CO_2 (right side, each comparison). The monoclonal C219 was used to detect MDR protein. Some variability in overexpression is observed for all cell lines in different experiments; thus we show an example of dramatic (SW620; about 10-fold), poor (HCT15; about 3-fold), and moderate (SKRC-39; about 4-fold) overexpression. Mean fold overexpression at 5 days' growth in 10% CO_2 for SW620, HCT15, and SKRC-39 was about 7-, 4-, and 5-fold, respectively ($10 > n > 3$). Note that reactivity of anti-p-GP antibodies to low molecular weight bands (particularly near 40 kDa and near 25 kDa) is almost always seen in western blots as performed in a number of different laboratories (Meyers *et al.*, 1989; Tanaka *et al.*, 1990; Lincke *et al.*, 1990; Al-Shawi & Senior, 1993), including our own. These additional bands may represent proteolytic products or some intrinsic cross reactivity of C219. We did find that lack of serine protease inhibitors during isolation of membrane fragments leads to an increase in the low molecular weight reactivity for some preparations (not shown).

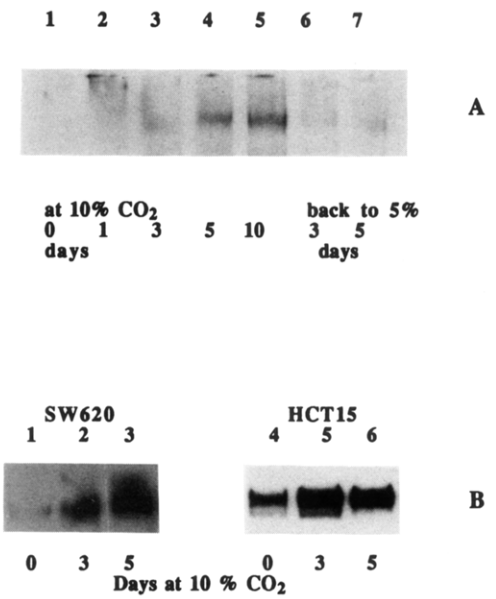

FIGURE 4: Western blot analysis of the time course of MDR overexpression in SW620 cells upon acid shock. Each lane was loaded with 20 μg of membrane protein. (A) Lane 1 = SW620 cells grown at 5% CO_2; lanes 2, 3, 4, 5 = SW620 cells grown at 10% CO_2 for 1, 3, 5, and 10 days, respectively; lanes 6, 7 = SW620 cells grown at 10% CO_2 for 5 days and then shifted back to 5% CO_2 for 3 or 5 days, respectively. For lanes 5–7, cells grown at 10% CO_2 for 5 days were harvested by trypsinization and then replated. (B) A western blot of SW620 was overexposed so that relative fold overexpression at 3 and 5 days could be compared for SW620 and HCT15. Lanes 1, 4 = SW620, HCT15 grown at 5% CO_2; lanes 2, 5 = SW620, HCT15 grown at 10% CO_2 for 3 days; lanes 3, 6 = SW620, HCT15 grown at 10% CO_2 for 5 days. In both A and B the monoclonal antibody C219 was used to detect MDR protein.

days' exposure to butyrate (Mickley *et al.*, 1989), but similar to the overexpression seen at 24 h [see Figure 5, Mickley *et al.* (1989)].

We also note some variability in these results. Although we have performed these experiments dozens of times, and although we consistently see elevated expression upon growth at 10% CO_2, with SW620 cells for example the fold increase calculated at 5 days has varied between 4- and 12-fold in different experiments (n = 6; mean = 7-fold, see Figure 3 caption). Thus in Figure 3 we show an example of robust overexpression for SW620, an example of poorer overexpression for HCT15, and an example of moderate overexpression for SKRC-39 to highlight this variability, which may be due to mild unavoidable variability in pH_o during acid shock at 10% CO_2.

Figure 4A,B shows the time course of increased MDR protein expression for the SW620 and HCT15 cells. In Figure 4A, note that increased expression for SW620 approaches maximal levels after 5 days' growth at 10% CO_2, with only a mild increase seen upon another 5 days' growth in elevated CO_2 (compare lanes 4 and 5). In contrast, when the cells grown at 10% CO_2 for 5 days are then shifted back to a 5% CO_2 atmosphere, increased expression is reversed within 3 days (compare lanes 4 and 6). By 5 days' growth at 5% CO_2 increased expression has returned to near basal levels (compare lanes 1 and 7). In Figure 4B we compare the increased expression seen at 3 and 5 days' growth at 10% CO_2 for the SW620 and HCT15 lines vs MDR protein expression for both cells grown at 5% CO_2. Although absolute levels differ (see caption), fold overexpression and the time course of overexpression are similar.

Notably, we are unable to detect increased MDR protein expression for acid-shocked human myeloma cells (RPMI 8226), Balb/c mouse 3T3 cells or chinese hamster lung fibroblast (DC-3F) or ovary fibroblast (LR73) cells in similar experiments (data not shown).

Acid Shock Elevates MDR mRNA. To test whether these data indicate a pH-dependent increase in the "membrane lifetime" of existing protein or highlight an increase in gene expression, we isolated RNA from the acid-shocked SW620 cells and probed for MDR mRNA using a 0.7-kbp hu MDR 1 specific fragment. Figure 5A shows a representative northern blot, and Figure 5B plots relative expression after scanning the blot with a Betagen imager and normalizing the MDR signal to the signal for β-actin (Roepe *et al.*, 1993; similar data are obtained upon normalizing to the signal for rPO). We find that MDR mRNA is increased in the acid-shocked cells, with 3–4-fold higher levels seen within 1 days' growth at 10% CO_2 in various experiments. Moreover, the increased levels of mRNA persist for as long as the acid burden remains, neither increasing further nor decreasing significantly, indicating that the cells do not "adjust" to the acid shock and then lower MDR mRNA back to normal levels, nor continue to overexpress mRNA within the experimental time course. Notably, although the mRNA overexpression is mild, this increase is seen in several blots using several different RNA preparations (not shown). In contrast, we do not find evidence for overexpression of other mRNAs, including, surprisingly, that encoding the Na^+/H^+ exchanger (even upon probing purified mRNA; data not shown) which acts to alkalinize many cells (we do, however, measure slightly increased Na^+/H^+ exchanger activity in these cells; see Discussion).

Acid-Shocked Cells Exhibit an MDR Phenotype. We next tested whether the acid-shocked cells expressing increased MDR protein were multidrug resistant, relative to the control

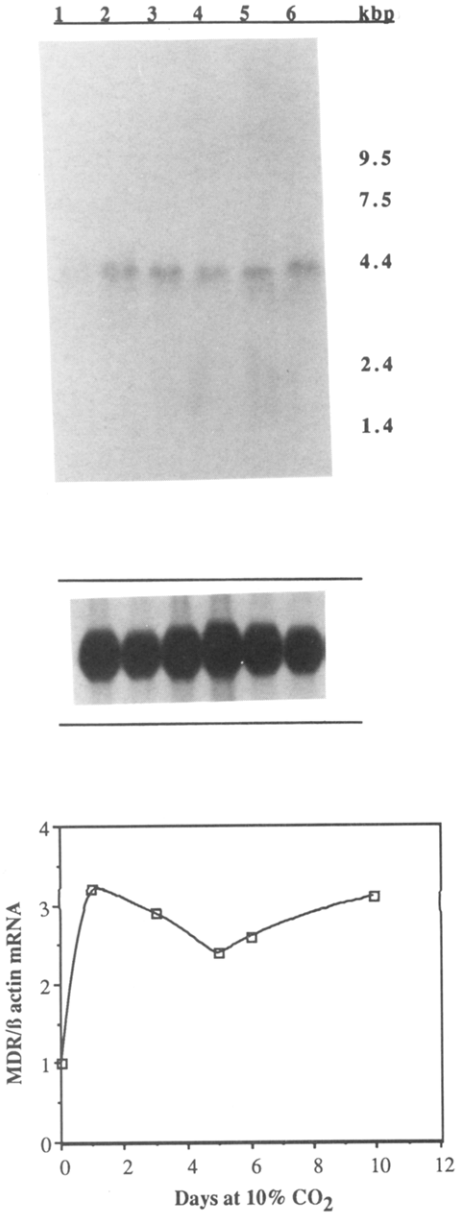

FIGURE 5: Northern blot analysis of hu MDR 1 mRNA levels using a gene specific cDNA probe (see Methods). (A, top) Representative northern blot of total RNA isolated from (from left) SW620 grown at 5% CO_2, or grown at 10% CO_2 for 1 (lane 2), 3 (lane 3), 5 (lane 4), 6 (lane 5), or 10 (lane 6) days. Similar data was obtained in other northern blots using other RNA preparations (not shown). β-Actin signals from the same blot, shown at the bottom, reveal that β-actin levels do not change dramatically upon acid shock. (B, bottom) A plot of relative expression of the MDR mRNA after normalization of the signal to that for β-actin (similar data is obtained by normalization to rPO). By quantitating β-actin and rPO vs ribosomal RNAs, we determined that acid shock conditions do not lead to significant alterations in the relative level of β-actin (see bottom of this figure) or rPO mRNA (not shown). Note that relative overexpression reaches a maximum at 3 days and then remains relatively constant. Not shown is the return to normal mRNA levels when cells are shifted back to 5% CO_2 for several days (but see Figure 4).

cells. Thus, induced and uninduced SKRC-39 cells were assayed for relative resistance to doxorubicin (Figure 6A), vincristine (Figure 6B), colchicine (Figure 6C), and methotrexate (Figure 6D) by a cytotoxicity assay. Importantly, both induced and uninduced cells were incubated with drug at 5% CO_2 for a short period of time (i.e., well before MDR induction is reversed, see Figure 4) in preequilibrated media and subsequently grown for 3 days in a 5% CO_2 environment.

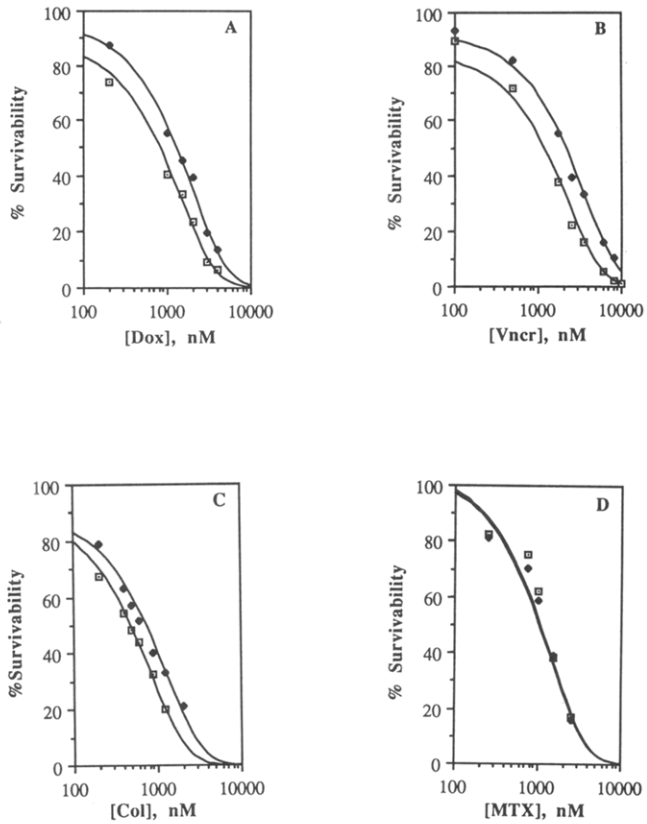

FIGURE 6: The results of cytotoxicity assays for acid-shocked SKRC-39 cells (solid tilted squares) and control SKRC-39 cells grown at 5% CO_2 (open squares) vs various concentrations of (A) doxorubicin, (B) vincristine, (C) colchicine, or (D) methotrexate (see Methods). Note that the acid-shocked cells are resistant to the drugs in the "MDR spectrum" of chemotherapeutics, but not to the antifolate. For each assay, three individual determinations of relative survivability were made by plating the cells in triplicate; these data were then averaged. Data shown in these plots are the averages of three different assays (i.e., nine individual determinations). Each curve is fitted by an exponential ($R^2 > 0.90$ for each curve). Note that similar data are also obtained for HCT-15 cells (not shown).

Thus, relative resistance is not due to different pH_o during exposure to drug.

In any case, the overexpressing SKRC-39 cells are clearly resistant to the antineoplastics in the "MDR spectrum" of compounds (A–C), but not to methotrexate (D). Also, the mild resistance is reversed by >75% upon coincubation with 10 μM verapamil (not shown). Incubation with the same concentration of verapamil alone has no effect on cell survivability. In contrast to these data, we find no evidence for a MDR phenotype for a variety of cell types grown at 3% CO_2 (S. Basu and P.D.R., unpublished) which "alkaline shocks" the cells.

As an additional test of the MDR phenotype, retention of [^{3}H]vinblastine vs time was assayed for induced and uninduced cells (Figure 7). The induced cells retain about 20% less vinblastine over a 20-min time period. Thus, in several key respects, these cells appear to behave similarly to MDR cells generated either by selection with increasing concentrations of chemotherapeutics or by transfection of hu MDR 1 cDNA (Hammond *et al.*, 1989).

Volume Perturbations also Induce MDR Expression. Since alterations in pH_i are often seen during the RVD or RVI response associated with osmotic shock and since some proteins involved in pH_i homeostasis are also involved in volume regulation, we tested whether osmotic shock altered the level of MDR protein expression.

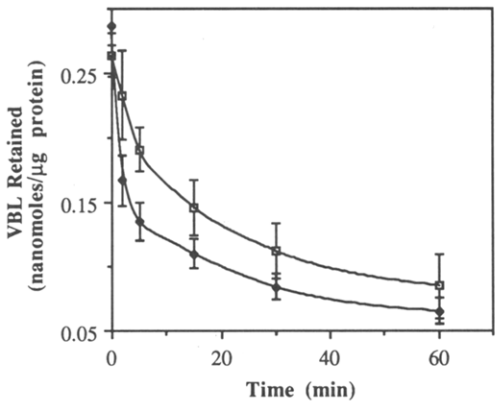

FIGURE 7: Retention of [^{3}H]vinblastine over time for SW620 cells grown continuously at 5% CO_2 (open squares) or 10% CO_2 for 5 days (solid tilted squares). Note that although they are preloaded to essentially the same level of [^{3}H]vinblastine, the acid-shocked cells retain considerably (~20%) less chemotherapeutic drug over time. Each point is the average from three separate experiments. See Methods for additional experimental details.

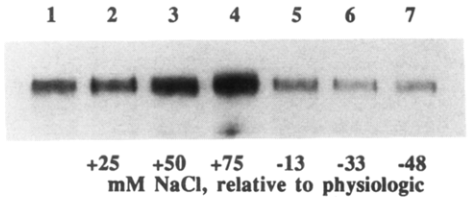

FIGURE 8: Relative expression of MDR protein in membranes isolated from colon cells subjected to hypertonic (lanes 2–4) or hypotonic (lanes 5–7) stress as assayed by western blot with the monoclonal C219. Cells were grown under normal culture conditions and were then split into seven flasks that could be loosely capped to prevent evaporation without inhibiting exchange of CO_2. After 4 days' growth the cells were harvested and membranes were isolated as described. Lane 1 is a membrane fraction from cells grown in normal media, lanes 2–4 are membranes from cells grown in the same medium plus additional 25, 50, or 75 mM NaCl, respectively, and lanes 5–7 are membranes from cells grown in 10 mL of media plus an additional 1, 3, or 5 mL of water (i.e., 132, 112, or 97 mM NaCl). Note the increase in expression for the hypertonically shocked cells, but no increase for the hypotonically shocked cells. Similar data was also obtained for the SKRC-35 kidney cells (not shown).

Figure 8 shows a western blot of membranes prepared from colon carcinoma cells grown in normal media (lane 1), media plus additional salt (hypertonic, lanes 2–4), or media plus additional water (hypotonic, lanes 5–7) for 4 days. We find that hypertonic shock, similar to acid shock, induces overexpression of MDR protein within this time frame. Increasing the hypertonicity promotes a greater increase in expression (compare lanes 2–4). Thus, cells grown in the presence of additional 75 mM NaCl (i.e., 220 mM NaCl overall, lane 4) express about three to four times more MDR protein than cells grown in the presence of additional 25 mM NaCl (lane 2). Conversely, hypotonic shock, which has been found to stimulate Cl^- translocation by the MDR protein (Valverde *et al.*, 1992; Gill *et al.*, 1992), does not appear to increase levels of MDR protein in the membrane (lanes 5–7) even upon reducing extracellular NaCl to 97 mM (lane 7). In fact, in several experiments it appears as if a very slight decrease in protein levels occurs under these conditions (Figure 8). Similarly, hypertonic (additional 75 mM NaCl) but not hypotonic shock is found to increase MDR protein expression about 5-fold for the SKRC-39 cells (not shown). The hypertonically shocked cells, in analogy to the acid-shocked cells, are also found to exhibit mild resistance to various chemotherapeutics (data not shown).

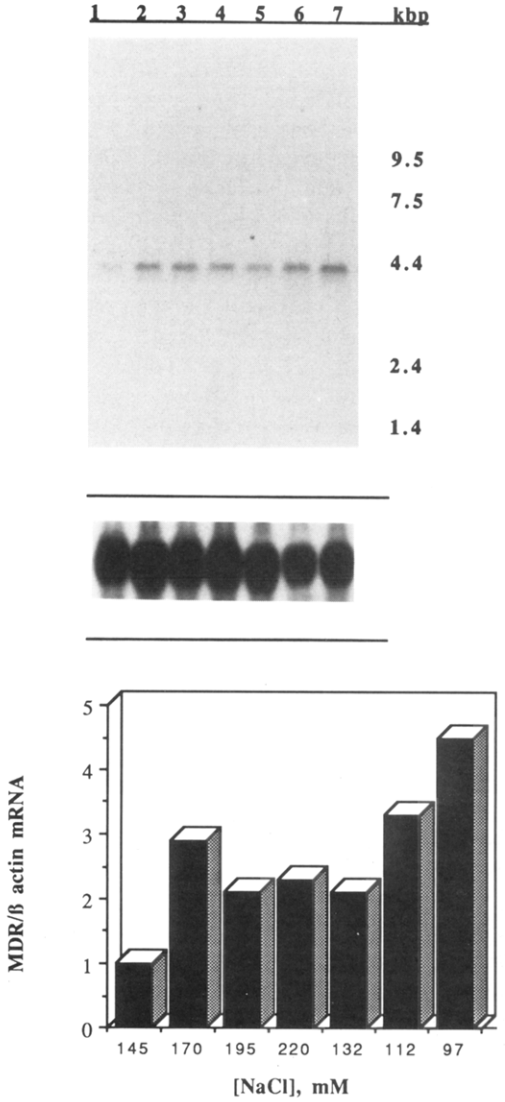

FIGURE 9: Northern blot analysis of hu MDR 1 mRNA levels for colon cells subjected to either hypertonic (lanes 2–4) or hypotonic (lanes 5–7) conditions. (A, top) Representative northern blot (similar data are obtained with other blots using other RNA preparations) that also show β-actin signals for the same blot (at bottom). Lane 1 is RNA from cells grown for 4 days under standard culture conditions, lanes 2–4 are RNA from cells grown in medium plus 25, 50, and 75 mM additional NaCl, respectively, and lanes 5–7 are RNA from cells grown in 132, 112, or 97 mM NaCl (10, 23, or 33% hypotonic), respectively. Control experiments using NaCl-free but otherwise complete media to alter tonicity reveal that these effects are unlikely to be due to dilution of vitamins, amino acids, etc. (B, bottom) Quantitation of MDR mRNA after normalization to the signals for both β-actin and acidic ribosomal phosphoprotein (rPO). Data shown is the average of four separate quantitations using separate northern blots and separate RNA preparations from separate cell cultures. As described in the caption to Figure 5B, we also measured the level of β-actin and rPO mRNA relative to ribosomal RNA, and note that the most dramatic hypertonic and hypotonic conditions *lower* these mRNAs by 20–40% in some experiments (see also bottom of Figure 9A); however, quantitation using either control mRNA is similar.

To examine whether the increased expression in these cells is also due at least in part to increased levels of MDR mRNA, we performed quantitative northern blot analysis as described previously for the acid-shocked cells. Figure 9 demonstrates that, surprisingly, *both* hypertonic *and* hypotonic conditions lead to increased levels of MDR mRNA in the SW620 colon cells. The levels of overexpression are similar to those seen upon acid shock (compare Figures 5A and 9A), and they follow a similar time course (not shown).

DISCUSSION

In prokaryotes such as *Escherichia coli*, adaptation to lower pH_o is known to include induction of the *exaR* gene, which is believed to regulate lactate and alcohol dehydrogenases as well as fumarate reductase (Auger *et al.*, 1989; Slonczewski, 1992). The low-pH_o adaptive response is less well studied for many eukaryotic cells. Since solid tumors generally grow in acidic environments (Wike-Hooley, 1984; Jain *et al.*, 1984), understanding acid induction of various genes, particularly genes involved in determining a tumor cell's response to cancer therapy, may be of considerable value.

Also, since elevated pH_i is a key feature of many MDR tumor cells that overexpress MDR protein, we wondered if in some cases increased MDR protein expression might form part of an adaptive response to acid pH_o, which would at least transiently lower pH_i. This would have obvious implications for solid tumors growing in acid environments, since some curiously exhibit MDR and increased MDR protein or mRNA levels prior to exposure to chemotherapeutic drugs.

In this report we have described our initial observations pertaining to these questions:

1. Growth of kidney or colon carcinoma cell lines at elevated (10%) CO_2, which lowers pH_o by about 0.5 units, leads to an increase in MDR protein levels within 3–5 days. The time course of overexpression matches that for recovery of pH_i, initially lowered by acid pH_o. After 5 days increased levels remain constant, but overexpression is reversed when cells are shifted back to 5% CO_2. This reversal is correlated with reversal of the alkaline "pH_i overshoot" phenomenon (Figure 2). Exposing other cells that have undetectable or extremely low levels of MDR protein to acid pH_o does not lead to increased MDR expression within the same time period. Thus, although our current analysis is by no means comprehensive, since endogenous physiologic expression of MDR protein is highest in colon, kidney, liver, and adrenal tissue, it is possible that acid induction only occurs for cells that normally express measurable amounts of the protein, i.e., for cells where MDR function is part of normal physiology.

2. Increased MDR protein is likely due, at least in part, to increased MDR mRNA. Current levels of sensitivity do not allow us to unequivocally conclude if the observed increased levels of MDR protein can be completely accounted for by increased levels of mRNA (assuming similar rates of translation and post-translational events). However, with our current level of detection and considering the mild variability in protein overexpression we observe (see above), it is conceivable that this is the case. Thus, any postulated increase in the plasma membrane stability or lifetime of MDR protein under acidic conditions does not likely account fully for the increased protein levels.[2]

3. The acid-shocked cells exhibit a "typical" MDR phenotype in that they are MDR, they retain less vinblastine, and resistance is reversed by verapamil.

4. Hypertonic shock also increases MDR protein and mRNA in the colon and kidney lines. Conversely, hypotonic shock increases MDR mRNA, but not MDR protein. We have no simple explanation for the curious observation that hypotonic conditions lead to increased mRNA without a concomitant increase in protein; however, we speculate that additional events that occur upon hypotonic shock might inhibit efficient MDR protein synthesis or membrane insertion. Possible explanations that merit further study include (i) MDR mRNA from the hypotonically shocked cells is less efficiently translated due to any of a variety of reasons, (ii) hypotonic conditions might affect the half-life of the MDR protein, and (iii) hypotonic conditions, which likely stimulate Cl^- transport by existing MDR protein (Valverde *et al.*, 1992; Gill *et al.*, 1992), might make increased levels of new protein deleterious.

Many tumors exhibit MDR without prior exposure to chemotherapeutics (Kakehi *et al.*, 1988; Baker *et al.*, 1990; Goldstein et al., 1992). Some "intrinsically MDR" tumor cells, particularly of colon, kidney, and liver origin, express measurable amounts of MDR protein. Although the differentiation agents DMSO or butyrate (Mickley *et al.*, 1989) and heat shock or exposure to arsenite (Chin *et al.*, 1990) have been shown to increase MDR protein expression in some cells, the mechanism responsible for overexpression of MDR protein in intrinsically MDR tumor cells is currently unclear. On the basis of the data presented in this paper, low pH_o induction may provide a satisfying answer, since low pH_o is a common yet unique environmental parameter for many solid tumor cells.

Since we do not observe the dramatic changes in cell morphology or growth rate previously observed for these colon cells upon exposure to differentiation agents (Mickley *et al.*, 1989) it is unlikely that low pH_o or hyper/hypotonic conditions induce a differentiation-like response in these cells that then causes overexpression. However, this point requires additional investigation.

Relatedly, however, we are not aware of any studies that have linked pH_o or volume perturbations to differentiation of *any* cells except in the particular case of gastric mucosa (Watanabe *et al.*, 1985). However, pH_i changes accompany differentiation of many cells (Larsson *et al.*, 1989; Ladoux *et al.*, 1989; Hazav *et al.*, 1989) so we do not exclude the possibility that the mechanisms for induction may overlap.

In any case, these results also differ from those reported previously (Mickley *et al.*, 1989) in that acid-shocked SW620 cells appear to retain lower levels of [^{3}H]vinblastine, but butyrate-treated cells apparently do not, even though they overexpress MDR protein even more [a recent study has suggested that this may be reversed by hyperphosphorylation of MDR protein; see Bates *et al.* (1992)]. Although differences in the cultured lines could be invoked as an explanation, we also suggest that acid-induced overexpression provides additional factors that more fully promote a MDR phenotype. One factor might be induction of Na^+/H^+ antiport activity, since we have associated this with the MDR phenotype in other cells (Roepe *et al.*, 1993) and since we find mildly increased Na^+/H^+ antiport activity in the acid-shocked cells (data not shown). Another explanation could be a post-translational modification of MDR protein necessary for function that is inhibited by differentiation as previously suggested (Mickley *et al.*, 1989), a likely candidate apparently being phosphorylation (Bates *et al.*, 1992).

Other recent studies have shown that increased MDR expression may occur in kidney carcinoma upon heat shock or exposure to arsenite (Chin *et al.*, 1990). Notably, however, these studies did not uncover increased MDR 1 expression for heat-shocked colon carcinoma cells. In contrast, we find increased expression in both cell types upon acid shock. Nonetheless, since heat shock is associated with a "stress

[2] Notably, previous determinations of the half-life of MDR protein indicate that it is quite long [16–18 h in DC3F-ADX cells (Meyers & Mendelsohn, 1991) and 12–17 h for human leukemia and ovarian cell lines (Muller & Ling, 1992)]. Thus, the lifetime spans the duration of the cell cycle, as measured in tissue culture. Since the doubling time of these cells is 12–14 h (cf. Figure 1), it is unlikely that increased lifetime of the protein alone would explain these data.

response", perhaps similar mechanisms may be involved in the stress response associated with volume or pH_o perturbations. This might be somewhat analogous to similar gene expression patterns observed in some bacteria upon exposure to different types of stress, including heat and acid shock. Further analysis of heat shock (or stress-associated) proteins may illuminate this point.

However, perhaps relatedly, hypertonic conditions are known to induce overexpression of HSP 70 in some eukaryotic cells (Cohen *et al.*, 1991). Thus, for some tissues, similar gene expression patterns may exist for both osmotically shocked and heat-shocked cells. The data in this and a previous paper (Chin *et al.*, 1990) on renal carcinoma cells is consistent with this idea.

Although increased expression of a protein by cells exposed to a certain environment is not proof that the protein plays a role in adaptation to that environment, since elevated pH_i has been observed in many different MDR cells and correlated with relative MDR protein overexpression (Keizer & Joenje, 1989; Roepe, 1992; Luz *et al.*, 1994), it is easy to envision a physiologic function for the overexpressed MDR protein in the acid-shocked cells, namely, that it perhaps participates in reequilibrating (i.e., elevating) pH_i. The agreement between the time course of induction and reequilibration of pH_i in the acid-shocked cells is consistent with this idea. It is important to point out, however, that currently we are unable to conclude if pH_i changes are directly or indirectly responsible for overexpression of MDR.

The role that increased MDR protein expression might be playing in the hypertonically shocked cells is more difficult to envision, but examination of other genes known to be induced by hypertonic shock may provide some insight (Burg & Garcia-Perez, 1992). In renal medullary cells these include genes encoding aldose reductase (which synthesizes sorbitol) and the betaine, taurine, and inositol transporters. Since sorbitol, taurine, betaine, and inositol are principle osmolytes of these cells, the effect of increased expression of these genes is increased intracellular organic osmolyte (synthesis or uptake), which increases V_i and thus decreases the deleterious increased intracellular salt concentration that is a consequence of cell shrinkage. Currently favored models state that MDR protein functions to directly efflux large organic molecules, so increased expression of the protein under *hypertonic* stress is confusing in the context of these models since effluxing large organic molecules would work *against* recovery of V_i. However, the alternative model for MDR protein function (Roepe, 1992; Roepe *et al.*, 1993) states that it indirectly lowers intracellular chemotherapeutic drug by translocating Cl^- (Valverde *et al.*, 1992; Gill *et al.*, 1992), and/or perhaps other ions directly or indirectly (Luz *et al.*, 1994), which then impact on pH_i, volume, and $\Delta\Psi$. Since transport of intracellular salt is an appropriate response to decreased V_i, this model more easily provides a physiologic rationale for hypertonic induction.

In summary, we have examined whether specific growth conditions of solid tumors (i.e., lower extracellular pH) might contribute to the development of the MDR phenotype in colon and kidney carcinoma cells that have not previously been exposed to chemotherapeutic drugs. We find that, as these cells adapt to the lower pH_o environment, they exhibit increased expression of MDR protein and other characteristic MDR traits. Hypertonic conditions appear to lead to similar overexpression of the MDR protein. Whether these represent general mechanisms for overexpression of MDR protein in other tissues remains to be determined; however, the results may have important implications for the study of multidrug resistance in some solid tumors.

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