Phenotypic Instability of Drug Sensitivity in a Human Colon Carcinoma Cell Line

Peter J. Ferguson and Yung-chi Cheng

ABSTRACT

Colon cancer is one of the tumors most refractory to treatment by chemotherapy, and this may be due to inherent phenotypic instability of such tumor cells with respect to the biochemical determinants of drug sensitivity. To test this hypothesis, a clonal human colon carcinoma cell line, clone A, was passaged in culture in the absence of selection conditions or mutagens. During this time, sensitivity to several drugs was examined, and was found to decrease 4-fold during 30 weeks of culture. Five randomly selected subclones, having never been exposed to drug or mutagen, displayed a range of sensitivities to etoposide (50% inhibitory concentrations ranging from 1.5 to 4.9 µM) and to vincristine (9-fold range), but all had the same sensitivity to methotrexate. With time these sensitivities also changed, and subsequent subclones were chosen from the lines with highest and lowest drug sensitivity. Again a wide range of phenotypes was observed. Sensitivity to vincristine ranged 14-fold and to doxorubicin 3-fold. Several biochemical determinants of drug sensitivity had a broad range of expression between cell lines. Cellular accumulation of [3H]vincristine, as well as expression of multidrug resistance protein P170 and glutathione transferase activity all varied significantly between subclonal lines. This suggests that some human colon tumors may be phenotypically unstable with respect to drug sensitivity, and this could contribute to clinical resistance to chemotherapeutic compounds.

INTRODUCTION

Gastrointestinal cancers, the most common cause of cancer deaths in the world, have historically been unresponsive to chemotherapy (1), a statistic contributed to in large part by colorectal cancer. The 20% response rate of colon tumors to treatment with 5-fluorouracil has not been improved upon by new therapies, either single drug or combination (2). Previously untreated colorectal tumors have shown little or no response in recent trials using 5-fluorouracil in combination with cisplatin (3), cisplatin plus DOX (4), or methotrexate (5). Thus it appears that in order to improve the chemotherapy approach to treatment of colon cancer, it is important to identify modes of resistance in these tumors, whether inherent or acquired, and the mechanisms by which they may be overexpressed.

Tumor heterogeneity has been suggested to be an important contributing factor in augmenting the difficulty in chemotherapy treatment of cancer (6). Such heterogeneity, which may involve expression of drug resistance phenotypes, could result from inherent instability of the tumor, and in turn may contribute to the refractory nature of colon tumors. The membrane glycoprotein p170, which has been established as a significant factor in multiple drug resistance in a laboratory setting (7), has been shown to increase significantly as tumors become increasingly refractory to chemotherapy (8, 9). Expression of the mdr1 gene has been readily detected in normal and tumorous colon tissue (10). Elevated levels of glutathione, GST, and glutathione peroxidase (for review, see Ref. 11) have been shown or implicated to contribute to resistance to a variety of chemotherapy agents, including DOX (12, 13). Increased activities of GST (up to 3-fold increase) and glutathione peroxidase (2-fold increase) have been found in tumors of normal colon tissue compared with normal colon (14, 15), with no change in glutathione itself.

The very high levels of p170 and glutathione-metabolizing enzymes in artificially established, drug-resistant cultured cell lines generally occur at a very low frequency, and must be selected for in the presence of a toxic agent. If acquisition of such resistance mechanisms were to occur with a high frequency, it would cause greater difficulty in treatment of a tumor, and could be responsible for the refractory nature of colon cancer. This paper describes a colon tumor cell line which, having never been exposed to any cytotoxic agent or mutagen, spontaneously expressed decreased sensitivity to three different chemotherapy drugs. Clonal lines expressing varying degrees of resistance were established, and several features of multiple drug resistance were characterized.

MATERIALS AND METHODS

Cell Lines

HCT-clone A, hereafter referred to as clone A, was cloned from a tumor line, DLD-1, established from an adenocarcinoma of the sigmoid colon (16), and was generously provided to our laboratory by Lan-Bo Chen, Harvard University, Boston, MA. Clone A was maintained in RPMI 1640 supplemented with 5% fetal bovine serum and kanamycin (100 µg/ml), and was grown in a humidified atmosphere of 5% CO2 in air. Monolayer cultures were subcultured weekly by harvesting with pancreatin (250 µg/ml) and diluting into a new flask. After 26 weeks of culture, subclones were established by diluting clone A cells into 60-mm Petri dishes such that 6–8 colonies would grow from single cells. Five colonies were chosen randomly, and named A-1 through A-5, respectively. Drug sensitivity was characterized for each line, and those with the highest and lowest resistance (A-1 and A-3, respectively) were maintained in culture. All lines and subsequently established lines were banked at −70°C as soon as enough cells were available (within 4 weeks of plating the original single cells) and periodically thereafter. After 30 weeks, the selection of subclones was repeated for A-1 and A-3, and 2 of 5 subclones of each of these lines were chosen based upon highest and lowest drug resistance. After another 21 weeks, this selection procedure was repeated for subclones A-1-3 and A-1-5 (selected from A-1) and for A-3-2 and A-3-4 (selected from A-3), from which 1 of 26 clones was maintained (A-3-4-6). After 48 weeks in culture, lines A-1-3, A-1-5, A-3-2, and A-3-4 were restarted from frozen cells banked after 8 weeks of culture. It must be stressed that at no time, whether in the patient or in cell culture, have any of these lines been exposed to any chemotherapy drugs. All of the clone A sublines reported here, except for A-3-4-6, resembled the parental clone A in size and morphology (irregular shape, no protrusions, well-defined boundaries). The A-3-4-6 cells, although similar in size, were rounded or oval shaped.

Human nasopharyngeal carcinoma KB cells were obtained from the American Type Culture Collection, and were maintained as described previously (17). KB/VCR100 and KB/VCR1000 cells were selected by exposure to stepwise increases in vincristine concentration (18), and were maintained routinely in the presence of 100 nM or 1 µM vincristine, respectively.

Effects of drugs on cellular proliferation were assayed as described previously (17). Briefly, rapidly proliferating cultures at approximately
10^2 cells/25-cm² flasks were exposed to drug for 3 days, and final cell number was enumerated by using an electronic particle counter (Celsoscope, Particle Data, Inc., Elmhurst, IL). The increase in cell number was calculated as a percentage of untreated cells, and IC₅₀ was determined by interpolation of plotted data.

**Uptake of [³H]Vincristine**

Cellular accumulation of vincristine after a 4-h exposure to radiolabeled drug was assayed as described previously (17), with some alterations. A method different from that previously described was used to determine background content of the flasks. Twenty-five-cm² flasks of cells, previously cooled on ice, were exposed to ice-cold [³H]vincristine-containing medium by rapid inversion of the flask for 1 to 2 s. Medium was immediately aspirated, and cells were washed with 2 rinses of 5 ml of ice-cold phosphate-buffered saline (0.15 M NaCl-0.65 mM KH₂PO₄, pH 7.4). This procedure was repeated for each separate experiment, and the amount of cell-associated drug obtained from this exposure [1.85 ± 1.65 (SD) pmol/10⁶ cells, n = 27] was subtracted from the determination of cellular drug content for presentation of data.

**Enzyme Assay and Antibody Staining**

GST activity was assayed as described (19) with the use of 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione as substrates. Extracts of rapidly proliferating cultured cells were obtained by scraping cell layers and resuspending cell pellets in the following buffer: 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 50 µM phenylmethylsulfonyl fluoride. Pellets were frozen and thawed once, and then sonicated for 5 s. This preparation was centrifuged (12,000 x g, 4°C, 15 min), and the resulting supernatant was used for assessment of GST activity as well as for "Western" blotting. The membrane pellet obtained was resolubilized in the following buffer: 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10% (w/v) sucrose, 80 mM dithiothreitol, and 2% (w/v) sodium dodecyl sulfate (20). Protein concentrations were determined by Coomassie dye (Bio-Rad Laboratories, Richmond, CA). After boiling and cooling of membrane samples, 10 µl urea was added to give a final concentration of 3 M (20). Samples for Western blotting were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% acrylamide (21), with 6 M urea for membrane samples (20), and blotted onto nitrocellulose paper by modification of the procedure of Towbin et al. (22). Before antibody staining, the nitrocellulose paper was blocked by using 2% powdered milk in phosphate-buffered saline for 30 min at room temperature. The primary antibody for GST (23) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD). Anti-pl70 antibody C219 (24) was generously provided by A. J. Townsend and C. E. Myers (National Institutes of Health Cancer Institute, Bethesda, MD).

**Materials**

Drugs. DOX was purchased from Adria Laboratories, Columbus, OH. VP-16 was a generous gift of Bristol-Meyers Pharmaceuticals, Syracuse, NY. Vincristine was kindly provided by Eli Lilly & Co., Indianapolis, IN. Methotrexate was purchased from Lederle Laboratories Division, American Cyanamid Co., St. Louis, MO.

Alkaline phosphatase-conjugated anti-mouse antibody was purchased from Promega Biotec, Madison, WI. Enzyme-conjugated anti-goat antibody was obtained from Fisher Scientific, Raleigh, NC.

[³H]Vincristine was purchased from Moravek Biochemicals, Inc., Brea, CA, and was purified within 1 week of use by high-performance liquid chromatography, using an isocratic elution of 65% (v/v) methanol in 5 mM KH₂PO₄, pH 4.9. High-performance liquid chromatography-grade chemicals (KH₂PO₄ and methanol) were obtained from Fisher Scientific. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Nitrocellulose paper was obtained from Schleicher & Schuell, Inc., Keene, NH. Tissue culture materials were purchased from Grand Island Biological Co., Grand Island, NY, and Hazleton Research Products, Denver, PA (serum). Plastic ware was purchased from commercial sources.

**RESULTS AND DISCUSSION**

HCT clone A cells and subsequently derived subclones were maintained as a monolayer culture. This poorly differentiated cell line was established from a colon adenocarcinoma after surgical resection before the patient received any chemotherapy (16). In our hands, it grew as expected, with little or no cell death observed between passages. After 3 weeks, clone A was assayed for sensitivity to VP-16, an inhibitor of topoisomerase II that is also influenced by the MDR phenotype (18) and found to have an IC₅₀ of 1.5 µM (Table 1). This is somewhat higher than that observed for 3 other colon tumor lines (HCT-8, HCT-116, HCT-CX-1) (range, 0.1 to 0.2) (18). The sensitivity to VP-16 was subsequently assayed at the times given, and the IC₅₀ was increased with time as shown, up to at least 4-fold higher. Controls were conducted with each experiment to ensure that the drug was exhibiting full activity. The shape of the dose-response curves was always linear, and the angle became gradually flatter, although it did not reach a plateau at the concentrations examined (in the 0–60% inhibitory range) (data not shown). This suggested that the population was becoming increasingly heterogeneous, and so at 26 weeks, subclones were established. Five randomly chosen clones were found to have the range of sensitivities to VP-16 shown in Table 2, all less than that of the parent line. At the indicated time points, dose-response curves were obtained by using duplicate samples for each drug concentration, and IC₅₀ values were determined from interpolation of data. The mean generation time (22 h) of all lines was identical during the period over which all these results were obtained, indicating that the differences in cell lines were not dependent on passage number. As indicated in Table 2, sensitivity to VP-16 tended to change slowly with time in several lines, including A-1, in which a slight increase in vincristine resistance was also observed at 15 weeks.

**Table 1** Effect of culture period on sensitivity of clone A cells to VP-16

<table>
<thead>
<tr>
<th>Length of culture (wk)</th>
<th>IC₅₀ of VP-16 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>1.8</td>
</tr>
<tr>
<td>14</td>
<td>2.3</td>
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<tr>
<td>18</td>
<td>3.3</td>
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<tr>
<td>20</td>
<td>4.5</td>
</tr>
<tr>
<td>26</td>
<td>5.3</td>
</tr>
<tr>
<td>30</td>
<td>6.6</td>
</tr>
</tbody>
</table>

**Table 2** Effect of culture period on sensitivity of subclones A-1 through A-5 to VP-16

<table>
<thead>
<tr>
<th>IC₅₀ (µM)</th>
</tr>
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<tbody>
<tr>
<td>VP-16</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Wk 5</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>VP-16</td>
</tr>
<tr>
<td>A-1</td>
</tr>
<tr>
<td>A-2</td>
</tr>
<tr>
<td>A-3</td>
</tr>
<tr>
<td>A-4</td>
</tr>
</tbody>
</table>

* VCR, vincristine.
After 15 weeks, sensitivity of both lines to vincristine was significantly altered. The IC₅₀ against A-3 increased over 3-fold in 5 weeks, to 75 nM, and then to 100 nM by the end of 35 weeks in culture. During this time, the IC₅₀ for A-1 increased from 230 nM at 15 weeks to approximately 400 nM by 30 weeks. (Averages for the upper level of the vincristine IC₅₀, determined after 35 weeks, are given in Table 3.) Thus, at 30 weeks, another set of subclones was established, again chosen randomly without drug selection, to observe whether A-1 and A-3 represented mixed populations. The distribution of sensitivities of these lines to vincristine, in terms of their first assay with this drug, demonstrated that this was again very much the case (Fig. 1). Except for the parent line of each set (set = one column), the values given are from the first assay with vincristine. The value of 340 nM for clone A was an average of 3 experiments conducted between 28 and 30 weeks of culture, and was slightly lower than that of 500 nM reported by Dexter et al. (16), even though the assay was very similar. Although the average drug sensitivity of the second set of subclones (A-1-3, A-1-5, A-3-2, A-3-4) was relatively stable with time, the establishment and assay of a third set of subclones after 21 weeks demonstrated that these also very quickly became a mixed population, as shown in Fig. 1.

In Table 3, the drug sensitivity data are summarized, along with assays of several possible determinants of drug sensitivity. In general, among cell lines there were wide variations in sensitivity to vincristine and DOX, compared with stable sensitivity to methotrexate. The sensitivity to vincristine and DOX appeared to be grouped as low in the A-1 subset and high in the A-3 subset, with subclone A-3-4-6 exhibiting the greatest sensitivity to both drugs. Cellular uptake of [³H]vincristine correlated fairly well within groups, but upon closer examination some anomalies appear, with respect to both vincristine uptake and comparison between vincristine and DOX. In comparing A-1-3 with A-3, the average IC₅₀ of vincristine differed by 2-fold, whereas the level of drug accumulation was the same. As well, sensitivity of A-1-3 to vincristine was only slightly greater than that of A-3-3, whereas drug accumulation was 2-fold greater. Sensitivity to vincristine did not always correlate with sensitivity to DOX. Line A-3-2 was less sensitive to vincristine than A-1-3, but was more sensitive to DOX than this line.

These results suggested the presence of multiple determinants of drug resistance, including the membrane glycoprotein p170 which could decrease intracellular drug accumulation, and possibly some contribution by glutathione metabolism, which has been implicated in modifying cellular sensitivity to DOX (12, 13). The activity of GST varied greatly among the clone A subclones, and did not correlate well with drug sensitivity. Although line A-1-5 was twice as resistant to vincristine than was A-1-3, these lines shared the same IC₅₀ to DOX, even though A-1-5 had a higher GST activity. As well, the similar GST activities between A-3-2 and A-3-4-6 did not correlate with the great difference in IC₅₀ of DOX, although between these 2 lines, a difference in uptake of DOX (which was not measured) may have been an important factor. Most of these values for GST activity were below the range of 70–160 milliunits/mg found in colon tumor explants (26), although vari-

### Table 3 Comparison of several parameters related to drug sensitivity in clone A and subclones of clone A

<table>
<thead>
<tr>
<th>Line</th>
<th>Vincristine IC₅₀ (nm)</th>
<th>Doxorubicin IC₅₀ (nm)</th>
<th>Methotrexate IC₅₀ (nm)</th>
<th>[³H]VCR uptake (pmol/10⁶ cells)</th>
<th>GST activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone A</td>
<td>340 ± 66 (3)</td>
<td>510 ± 100 (3)</td>
<td>4.9 ± 0.14 (2)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A-1</td>
<td>352 ± 45 (6)</td>
<td>344 ± 48 (4)</td>
<td>3.7 ± 1.5 (5)</td>
<td>5.85 ± 0.62 (3)</td>
<td>58.4 ± 17.8 (8)</td>
</tr>
<tr>
<td>A-1-3</td>
<td>197 ± 43 (10)</td>
<td>252 ± 6 (3)</td>
<td>3.4 ± 1.9 (4)</td>
<td>9.68 ± 1.31 (3)</td>
<td>47.6 ± 21.9 (7)</td>
</tr>
<tr>
<td>A-1-5</td>
<td>413 ± 105 (11)</td>
<td>265 ± 33 (3)</td>
<td>3.5 ± 1.4 (7)</td>
<td>5.45 ± 1.41 (6)</td>
<td>73.6 ± 13.7 (7)</td>
</tr>
<tr>
<td>A-3</td>
<td>89 ± 25 (5)</td>
<td>94 ± 19 (4)</td>
<td>3.6 ± 1.5 (4)</td>
<td>10.1 ± 3.1 (3)</td>
<td>5.82 ± 1.27 (6)</td>
</tr>
<tr>
<td>A-3-2</td>
<td>242 ± 43 (6)</td>
<td>150 ± 18 (3)</td>
<td>5.0 ± 1.1 (4)</td>
<td>4.52 ± 0.22 (3)</td>
<td>13.6 ± 6.9 (7)</td>
</tr>
<tr>
<td>A-3-4</td>
<td>30 ± 15 (15)</td>
<td>88 ± 14 (3)</td>
<td>3.0 ± 1.0 (3)</td>
<td>27.0 ± 13.0 (6)</td>
<td>15.2 ± 0.54 (6)</td>
</tr>
<tr>
<td>A-3-4-6</td>
<td>1.3 ± 0.11 (2)</td>
<td>9.6 ± 1.0 (4)</td>
<td>6.2 ± 0.07 (2)</td>
<td>341 ± 100 (3)</td>
<td>15.9 ± 3.3 (3)</td>
</tr>
</tbody>
</table>

* VCR, vincristine.  
* Mean ± SD.  
* Single numbers in parentheses, number of experiments; range of numbers, culture period, in weeks, during which assays were performed.  
* ND, not determined.

![Fig. 1. Sensitivity of newly selected clone A subclones to vincristine. After clone A was maintained in culture for 26 weeks, clonal lines were established, and sensitivity to vincristine was assayed after 10 weeks of culture. After 30 weeks of culture, another set of subclones was established from clones A-1 and A-3. Between 4 and 8 weeks of culture, vincristine sensitivity was again assayed for the parental culture and the subclones. The final series of subclones, from lines A-1-3, A-1-5, A-3-2, and A-3-4, were established after 21 weeks, and drug sensitivity was assayed between 1 and 6 weeks of culture. The IC₅₀ of vincristine for each line was determined as described in "Materials and Methods."
ables such as site of origin and degree of differentiation may contribute to this difference. The glutathione system may also have been responsible for the changing sensitivity to VP-16, as a nontoxic concentration of buthionine sulfoximine, a specific inhibitor of glutathione synthesis, was found to enhance VP-16 toxicity by 3.6-fold (27). Table 3 clearly shows that (a) there was wide variability among the cell lines with respect to a number of significant parameters, and (b) no single parameter was responsible for differences in drug sensitivity.

Given the association of p170 expression with MDR and reduced drug uptake (24, 28), the level of this protein in membrane preparations was examined by staining Western transfers with antibody C219. Fig. 2 is representative of 3 separate experiments with similar trends. The preparation from A-1-3 stained more intensely than that of A-3, which was more sensitive to the cytotoxicity of vincristine, even though these lines had similar 4-h accumulation of [3H]vincristine. Staining of p170 was detectable at the same level in the KB/VCR100 lines (IC50 of vincristine, 150 nm) as in HCT lines A-1, A-1-5, and A-3-2, 3 lines in which drug accumulation was about the same. The p170 protein was barely detectable in A-3-4 and undetectable in A-3-4-6, in which uptake was much higher than in the other lines. However, with the exception of A-1-5 (in which the high GST activity may have contributed to resistance), staining intensity of p170 correlated more closely with drug sensitivity than with drug accumulation.

Verapamil has been shown to enhance sensitivity of colon cells to vincristine (29) and DOX (30) by up to 4-fold. Against clone A subclones A-1-5 and A-3-2, exhibiting highest and lowest resistance to vincristine, verapamil enhanced toxicity 100-fold and 20-fold, respectively (3 experiments with each, data not shown). This indicates that MDR could be one of the major contributory factors to the resistance of these cells.

Fig. 3 is representative of 3 Western blots with similar trends. The antibody used in this procedure (23) has been shown to specifically stain GST-4, the form of the enzyme associated with drug resistance which also exhibits organic glutathione peroxidase activity (13). The enzyme was readily detected in the parent line and varied considerably among the subclones. Between A-3 and its subclones, the intensity of staining correlated well with the level of GST activity. The staining level of A-1 was consistently much less than that of A-1-3 in 3 experiments, even though the activity was about the same, and A-1-5 stained much more darkly than any other line, as shown by the intensity of a 2-fold dilution of protein. The antibody staining suggests that the GST activity assayed may not have been totally accounted for by only GST-4.

The overexpression of the p170 protein along with the MDR phenotype, as well as enhanced expression of GST, may be a consequence of several different factors. Overexpression of p170 has been found to result from gene amplification (31, 32), and such amplification has given rise to coamplification of up to 5 other genes in a single cell line (33–35). Although coamplification of the dihydrofolate reductase gene along with mdr1 has been reported (36), there was no indication that this was the case in any of the clone A lines, based on sensitivity to methotrexate. Amplified genetic material has been shown to exist as homogeneously staining regions (35), double-minute chromosomes (37, 38), and submicroscopic episomal DNA (39), although extrachromosomal DNA-dependent resistance has been found to be unstable (37, 38). It has not yet been determined whether these clone A lines express any of these features, although the stability of their resistance in the absence of selection pressure argues against an extrachromosomal determinant.

A second possibility is that regulation of gene expression was altered. Enhanced expression of the glutathione-metabolizing system, in response to selection conditions, has been reported (40), and several glutathione-metabolizing enzymes are overexpressed in MDR MCF-7 human breast tumor cells (13), but this is not believed to be due to amplification of the genes involved (13). Overexpression of the mdr1 gene has been observed without amplification of the gene in colchicine-resistant KB cells (41) and in progressive patient leukemia (42), and altered control of mdr1 gene expression has also been found in cases where the gene has been amplified (33, 34).

A third way that cells could accumulate multiple copies of genes is by acquisition of multiple copies of the chromosomes which carry them. Such a phenomenon has been described for an undifferentiated lung tumor cell line, where chromosome number doubled, followed by progressive loss of most extra chromosomes (43). During periods when chromosome number was high, chromosomal translocations and deletions took place (43), which could lead to altered copy number and/or expression of given genes. In light of the hyperploidy of the Clone A cell line (16), such a phenomenon could occur in these cells with continued passage in culture. Work is presently under way.
to determine gene copy number and mRNA expression for the proteins involved.

It has so far not been possible to ascertain a source of selection pressure on these cells which would cause a shift in expression of the phenotypes observed, especially in a single direction. In the laboratory from which these cells were obtained, the clone A line was maintained in a 50:50 mix of RPMI 1640 and Dulbecco's modified Eagle's medium, supplemented with 5% calf serum, compared with RPMI 1640 and 5% fetal bovine serum in our hands. However, this medium had no effect on the drug sensitivity of 2 other colon lines over a period of at least 6 months, the moderately differentiated HCT-116, and highly differentiated HCT-CX.1-4 Thus the unstable phenotype may be associated more with undifferentiated cells. Maintenance of cells in 10% fetal bovine serum for up to 12 weeks did not alter the drug sensitivity of clone A subclones, nor did banking of samples at −70°C and reculture. Thus the alteration of phenotype appears to have been a spontaneous event, with a tendency toward increased expression of at least several proteins.

In summary, the poorly differentiated HCT cell line clone A rapidly enhanced its drug resistance with length of culture time, without exposure to any drugs or mutations. Randomly chosen subclones displayed a range of drug resistance, the level of which was lower in each line than the parent, but which also increased with time in culture, some beyond the level of the parent. In subsequent, random selections of subclones, the new lines displayed a wide range of resistance, both greater and less than that of the immediate parent. Parameters of cellular phenotype previously shown to be associated with drug resistance were found to be variable between cells having varying degrees of resistance. The level of p170 and GST activity were generally measured in this study. The ability of these parameters to multifactorial, probably involving more parameters than those measured in this study. The ability of these parameters to change with time to enhance the resistance of the cultured clone A cells, without obvious selection pressure, may contribute to the difficulty in clinical treatment of colon cancers.

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