Dihydrofolate Reductase Gene Amplification-associated Shift of Differentiation in Methotrexate-adapted HT-29 Cells

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Abstract. Postconfluent cultures of HT-29 cells form a heterogeneous multilayer of which >95% of the cells are undifferentiated. In contrast, when stably adapted to normally lethal concentrations of methotrexate (10^{-6}–10^{-5} M), they form a monolayer of gobletlike cells (Lesuffleur et al., 1990) which secrete large quantities of mucins and display a discrete brush border with the presence of villin, dipeptidylpeptidase-IV, and carcinoembryonic antigen. When adapted to even higher concentrations of methotrexate (10^{-4} and 10^{-3} M) there is a shift in the pattern of differentiation from gobletlike to dome-forming absorptive-like cells. These cells still display an apical brush border which expresses villin and dipeptidylpeptidase-IV, but no longer express significant levels of mucins and carcinoembryonic antigen. This shift of differentiation coincides with a sudden amplification of the gene coding for dihydrofolate reductase and an increased activity of the enzyme.

THE ability of certain human colon carcinoma cell lines to differentiate in culture into absorptive or goblet cells has been invaluable to the study of intestinal cell differentiation and function (for review see Rousset, 1986; Neutra and Louvard, 1989; Zweibaum et al., 1991). The cell line HT-29, established in 1964 by Fogh and Trempe (1975) has been particularly useful. Since the original observation (Pinto et al., 1982) that HT-29 cells, which are mostly undifferentiated in standard culture, differentiate into absorptive cells when glucose is replaced by galactose in the medium, a number of different laboratories have isolated differentiated HT-29 subpopulations and clones. Some are of absorptive type (Zweibaum et al., 1985; Wice et al., 1985; Fantini et al., 1986; Huet et al., 1987; Laboisse et al., 1988; Hafez et al., 1990), others are mucus secreting (Augeron and Laboisse, 1984; Huet et al., 1987; Lesuffleur et al., 1990, 1991; Hafez et al., 1990) others fluid transporting (Augeron and Laboisse, 1984; Fantini et al., 1986; Hafez et al., 1990). These subpopulations and clones share two general features. Firstly, the protocols used for their establishment all involve some kind of stress, like replacement of glucose by galactose (Pinto et al., 1982; Wice et al., 1985; Fantini et al., 1986; Dudouet et al., 1987; Huet et al., 1987), hexose deprivation (Zweibaum et al., 1985), or treatment with drugs such as sodium butyrate (Augeron and Laboisse, 1984; Wice et al., 1985), polyethylene glycol (Laboisse et al., 1988), methotrexate (Lesuffleur et al., 1990), hexamethylene bisacetamide (Hafez et al., 1990), or 5-fluorouracil (Lesuffleur et al., 1991). Secondly their phenotype remains stable when they are subcultured under standard conditions, i.e., in the absence of stress.

These observations can be explained in two different ways. One possibility is that the undifferentiated HT-29 cells are like the multipotent stem cells of the intestinal crypt (Cheng and Leblond, 1974), and that the various treatments trigger critical events which commit the cells to a particular differentiated state. The other possibility is that the emergence of the differentiated populations is the result of a selective process. This would be consistent with the observation that a small proportion of differentiated cells are observed within standard HT-29 cultures (Lesuffleur et al., 1990), and that the isolation of the differentiated lines is probably always associated with a substantial cell mortality.

Whatever the mechanism(s) involved, it should be noted that, in the experiments reported so far, the shift was always in the direction from mainly undifferentiated cells towards populations stably committed to one, or even two differentiated phenotypes (Huet et al., 1987). When HT-29 cells are adapted to increasing concentrations of the anticancer drug methotrexate (MTX)[1] they form a mixed population of absorptive and goblet cells when adapted to 10^{-7} M MTX, and of goblet cells exclusively at higher concentrations (10^{-6} and 10^{-5} M) (Lesuffleur et al., 1990). This sequence

1. *Abbreviations used in this paper*: CEA, carcinoembryonic antigen; DHFR, dihydrofolate reductase; DPP-IV, dipeptidylpeptidase-IV; IC_{50}, concentration of drug that causes a 50% inhibition of control cell growth; MTX, methotrexate; SI, sucrase-isomaltase; R_T, transepithelial resistance.

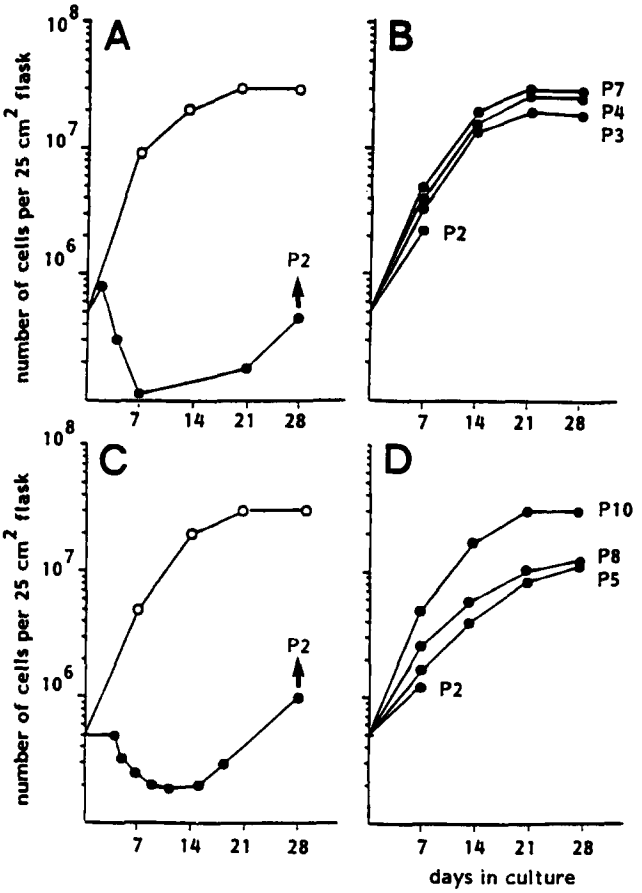

Figure 1. Growth adaptation of HT29-MTX10^{-5} cells to 10^{-4} and 10^{-3} M MTX. (*A* and *B*) Adaptation to 10-4M MTX. (*A*) Growth curve of HT29-MTX10^{-5} cells (*passage 11*) in the presence of 10^{-5} M MTX (○) and of the same cells cultured in the presence of 10^{-4} M MTX (●) and further subcultured after 28 d (*P2*). (*B*) Growth curves of following weekly passages (*P*) in the presence of 10^{-4} M MTX. (*C* and *D*) Adaptation of HT29-MTX10^{-4} cells to 10^{-3} M MTX. (*C*) Growth curve of HT29-MTX10^{-4} cells (*passage 7*) in the presence of 10^{-4} M MTX (○) and of the same cells cultured in the presence of 10^{-3} M MTX (●) and further subcultured after 28 d (*P2*). (*D*) Growth curves of following weekly passages (*P*) in the presence of 10^{-3} M MTX. Growth curves of HT29-MTX10^{-4} and HT29-MTX10^{-3} were stable after 7 and 10 passages, respectively.

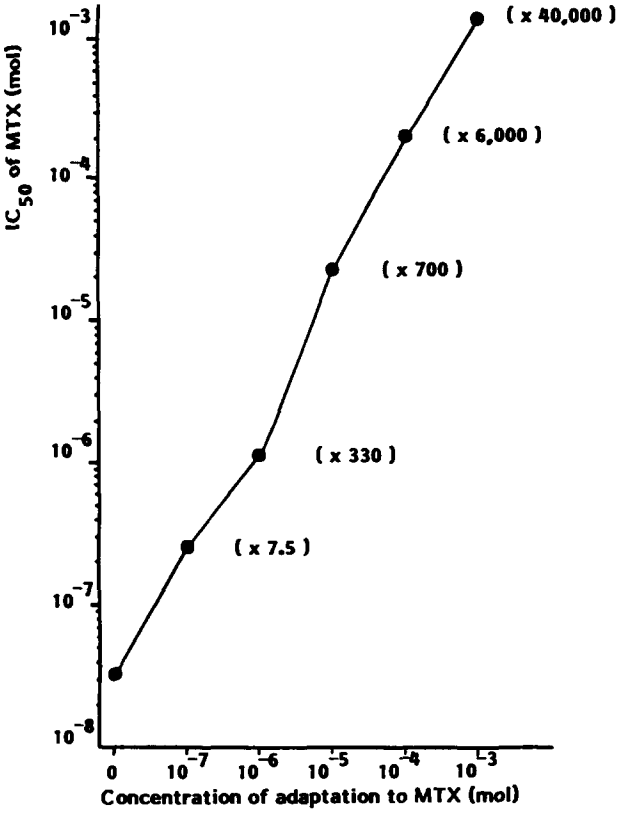

Figure 2. IC_{50} of methotrexate in parental and MTX-adapted HT-29 cells. Values are the mean of four to five determinations made at different passages of cells stably adapted to the concentrations of MTX indicated. SD (not shown) were <5%. Number in parentheses represent coefficient of increase as compared with the parental cells.

of events is compatible with a progressive selection process in which cells committed to both types of differentiation are resistant to 10^{-7} M MTX, whereas only cells committed to goblet cell differentiation are adaptable to higher concentrations.

The results reported here are in sharp contrast with this sequence of events. Indeed we show here that increasing the concentration from 10^{-5} to 10^{-4} and 10^{-3} M MTX unexpectedly results in a shift of differentiation from the goblet cell to the absorptive phenotype. We also show that this phenotypic change coincides with a major genetic event, namely a strong amplification of the gene coding for the target enzyme of MTX, dihydrofolate reductase (DHFR) (Jolivet et al., 1983).

Materials and Methods

Cell Culture

The parental HT-29 cell line, obtained from late Dr. J. Fogh (Sloan Kettering Memorial Cancer Center, Rye, NY), was used between passages 144 and 200 and is referred to as HT-29. Subpopulations adapted from 10^{-7} to 10^{-3} M MTX are referred to as HT29-MTX 10^{-n} when grown in the presence of MTX (Sigma Chemical Co., St. Louis, MI), and HT29-Rev MTX 10^{-n} when reversed to drug-free medium. Cells were grown in DME (Eurobio, Paris, France) supplemented with 10% inactivated (30 min, 56°C) FBS (Boehringer Mannheim Biochemicals, Mannheim, Germany). All experiments and maintenance of the cells were done in 25-cm^2 T-flasks (Corning Glassworks, Corning, NY) at 37°C in a 10% CO_2/90% air atmosphere. Cells were seeded at 5×10^5 cells per flask in all conditions. For maintenance purposes, cells were passaged weekly, using 0.25% trypsin in 0.53 mM EDTA in PBS Ca^2Mg^2-free. The medium was changed daily in all culture conditions.

Determination of IC_{50} of Methotrexate

Exponentially growing cells were seeded at 5×10^3 cells per well, in 96-well microtiter plates (Falcon, Oxnard, CA), in the presence of increasing concentrations of the drug. The medium was changed daily. Cell growth was assessed after 5 d as reported (Lesuffleur et al., 1990).

Differentiation Characteristics

Indirect immunofluorescence was performed on cryostat sections of cell layer rolls as reported (Lesuffleur et al., 1990). This method has the double

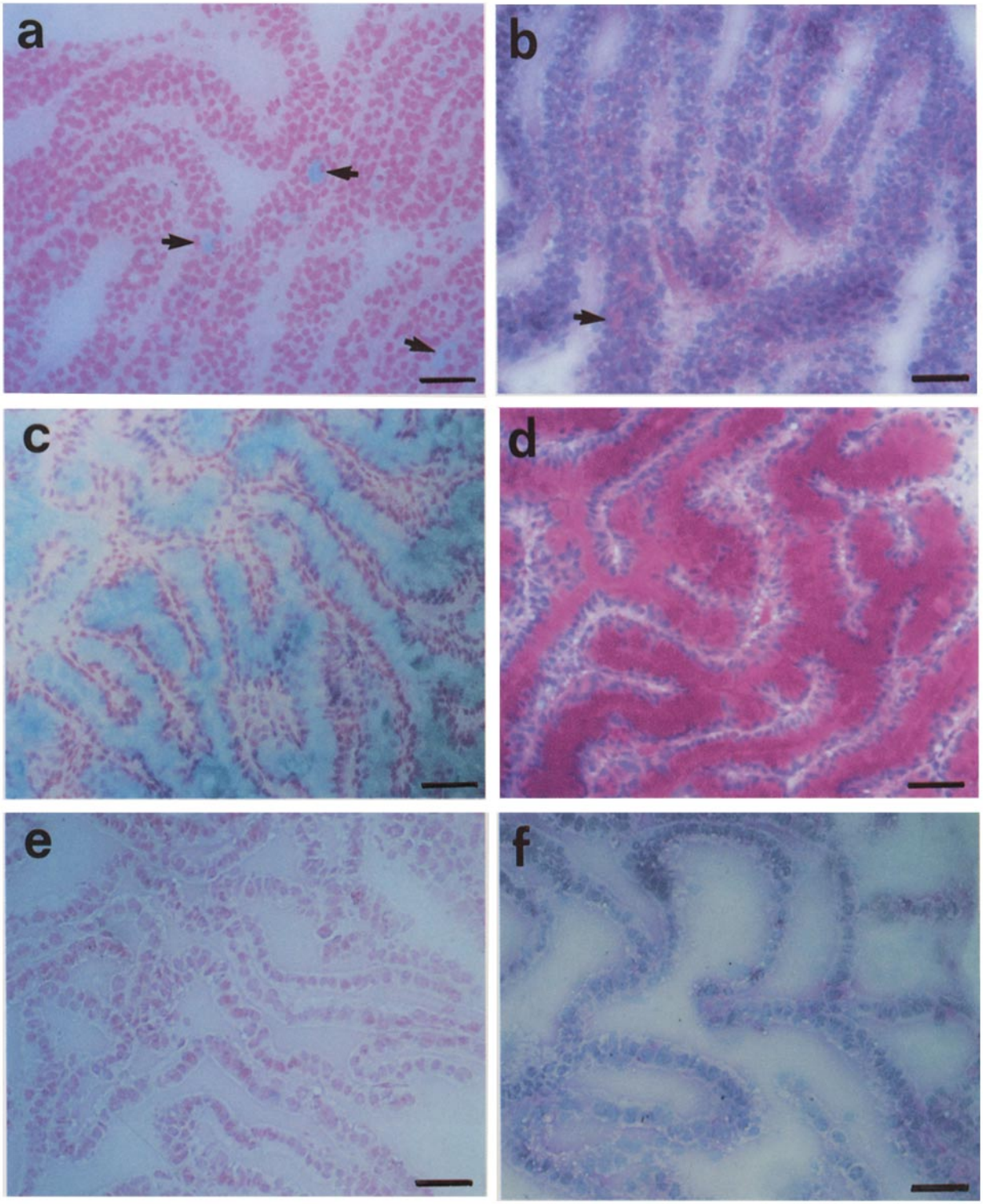

Figure 3. Cryostat sections of rolls of postconfluent cultures (*day 28*) of (*a* and *b*), parental HT-29 cells, (*c* and *d*), HT29-MTX10^{-5} cells (*passage 20*), and (*e* and *f*), HT29-MTX10^{-3} (*passage 17*) stained with alcian blue (*left*) and periodic acid Schiff (*right*). Note the multilayer organization of parental HT-29 cells and the presence of a few mucus-secreting cells (*arrows*). Note the monolayer organization of HT29-MTX10^{-5} cells with all cells secreting large amounts of mucins, but the total absence of mucins in HT29-MTX10^{-3} cells. The same results as observed in HT29-MTX10^{-3} cells were observed in cultures of cells adapted to 10^{-4} M MTX and at different passages (up to 20) of HT29-RevMTX10^{-4} and 10^{-3} cells (not shown). Bar, 68 μm.

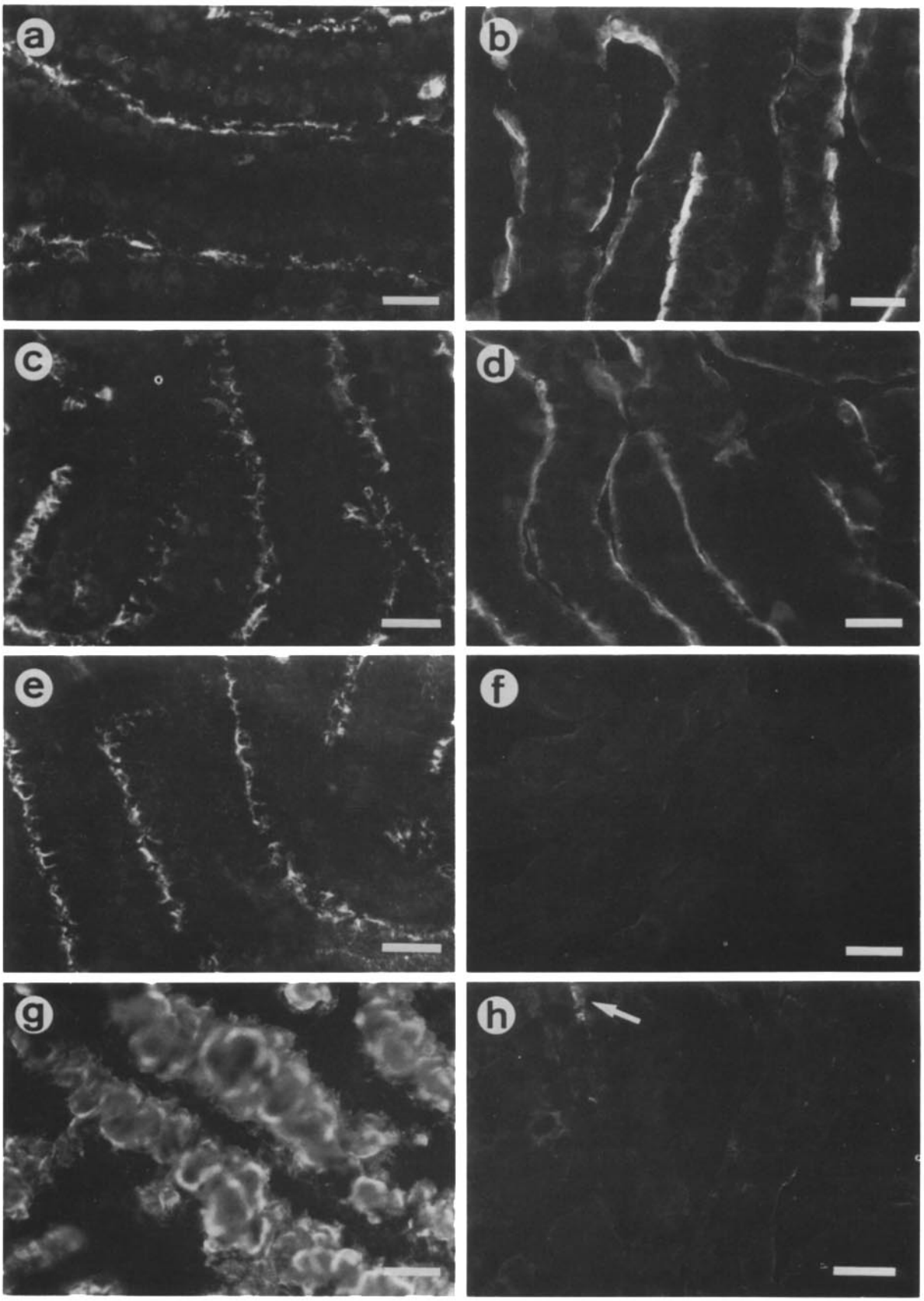

Figure 4. Indirect immunofluorescence staining of cryostat sections of rolls of postconfluent cultures of HT29-MTX10^{-5} (*left*) and HT29-MTX10^{-3} cells (*right*) with antibodies against villin (*a* and *b*), DPP-IV (*c* and *d*), CEA (*e* and *f*), and gastric mucins (*g* and *h*). Sections are from the same samples as in Fig. 3. Note the presence in HT29-MTX10^{-5} cells of an apical expression of villin, DPP-IV, and CEA and the large amount of mucins of gastric immunoreactivity. Note the apical expression of villin and DPP-IV, the absence of immunoreactive

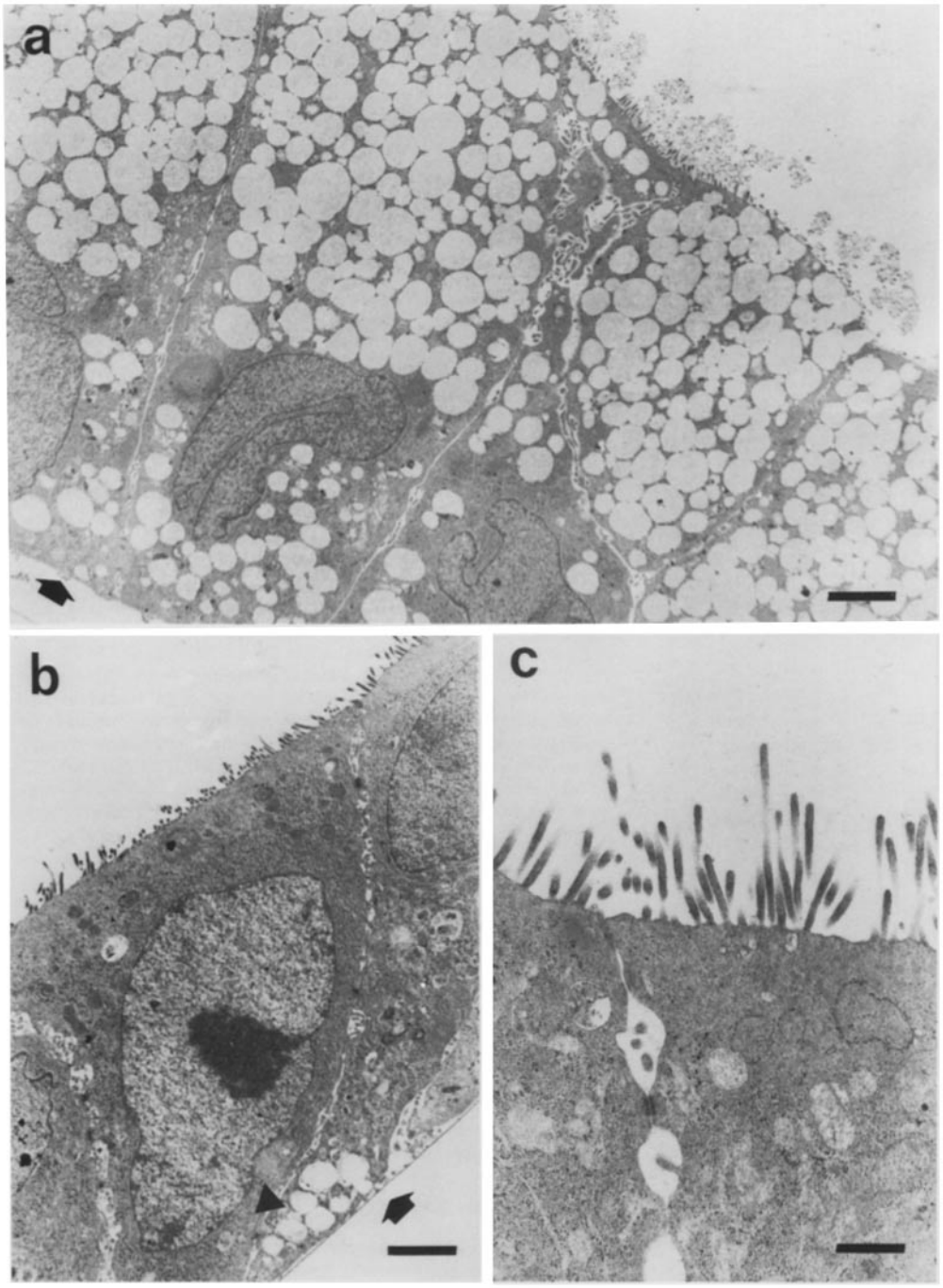

Figure 5. Transmission EM of postconfluent cultures (*day 28*) of MTX-adapted HT-29 cells. Sections are perpendicular to the bottom of the flask (*arrows*). (*a*) HT29-MTX10^{-5} cells (*passage 14*) showing a goblet cell organization with the presence, in the supranuclear compartment of numerous mucus droplets, and on the luminal border of a discrete brush border. (*b*) HT29-MTX10^{-3} cells (*passage 17*). Note the monolayer and polarized organization of the cells with the presence of an apical brush border and the absence of mucus secretions with only a very few cells, like one shown here, exhibiting a few mucus droplets (*arrow head*). (*c*) Higher magnification of the upper compartment of *b* showing details of the junctional complexes and of the brush border. Bars: (*a*) 2.7 μm; (*b*) 2.5 μm; (*c*) 0.9 μm.

advantage to visualize, on a same section, almost the totality of the cell layer and allow the detection of both apical and intracellular proteins. Sections were fixed with 3.5% paraformaldehyde in PBS Ca^2Mg^{32}-free (10 min, room temperature). Mouse mAbs HBB 3/775/42 and HBB 2/614/88, specific for human small intestinal dipeptidylpeptidase-IV (DPP-IV) and sucrase-isomaltase (SI), respectively (Hauri et al., 1985), were a gift from Dr. H. P. Hauri (Biocenter of the University of Basel, Basel, Switzerland). Rabbit polyclonal antibodies against porcine villin (Robine et al., 1985) were a gift from Dr. D. Louvard (Institut Pasteur, Paris, France). Polyclonal rabbit antibodies against mucins from gastric and colonic normal human mucosa were produced as reported (Lesuffleur et al., 1990). For the detection of the *P*-glycoprotein, product of the multidrug resistance gene (Riordaan et al., 1985) we used mAb MRK-16 (Hamada and Tsuruo, 1986), a gift of Dr. T. Tsuruo (Cancer Chemotherapy Center, Tokyo, Japan). mAb

CEA, and the presence of only trace amounts of gastric mucins (*arrow*) in HT29-MTX10^{-3} cells. The same results as observed in HT29-MTX10^{-3} cells were observed in cells adapted to 10^{-4} M MTX, as well as in HT29-RevMTX10^{-4} and HT29-RevMTX10^{-3} cells (not shown). No colonic mucin immunoreactivity was found in cells adapted to 10^{-5} MTX, as reported previously (Lesuffleur et al., 1990), nor to 10^{-4} or 10^{-3} M MTX (not shown). Bar, 30 μm.

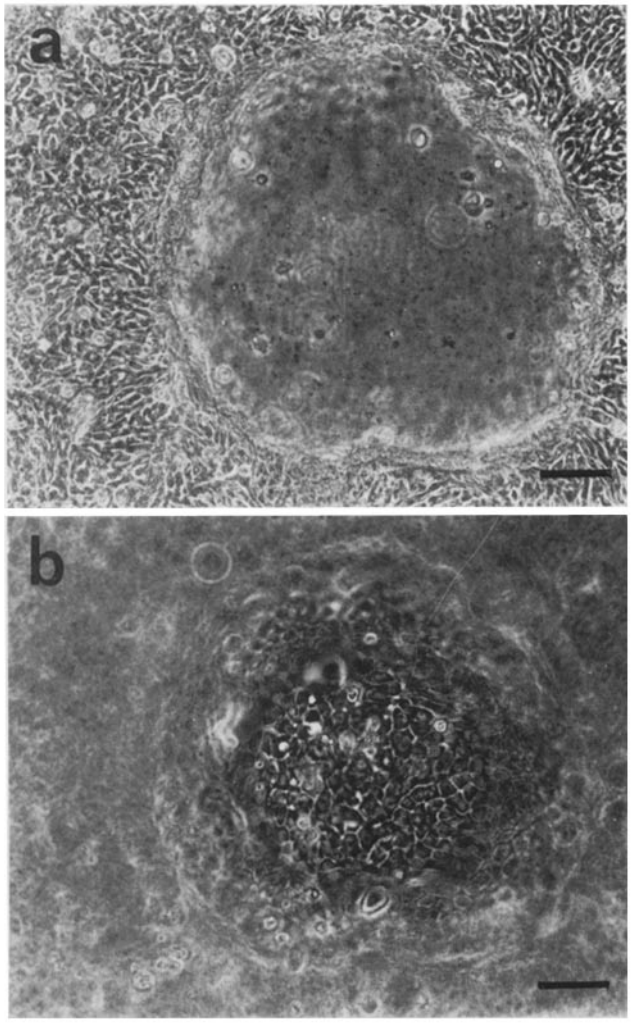

Figure 6. Phase contrast microscopy of postconfluent (*day 25*) HT29-RevMTX10^{-3} cells showing the presence of a dome. (*a*) Focused on monolayer. (*b*) Focused on top of dome. Bar, 70 μm.

601 against colonic carcinoembryonic antigen (CEA) was purchased from Biosys (Compiègne, France). Anti-rabbit fluorescein-coupled sheep antiglobulins were from Institut Pasteur Productions (Marne la Coquette, France). Anti-mouse fluorescein-coupled rabbit antiglobulins were from Cappel Laboratories (Cochranville, PA). Histological staining of cryostat sections with periodic acid Schiff and alcian blue (pH 2.5) was done as classical. Transmission EM was performed on cells grown in 25-cm^2 plastic flasks. Samples embedded in Epon were reembedded to make sections perpendicular to the bottom of the flask. The activities of DPP-IV and sucrase were measured in cell homogenates, using classical methods as previously reported (Chantret et al., 1988). Results are expressed as milliunits/mg protein. One unit is defined as the activity that hydrolyzes 1 μmol of substrate per min at 37°C.

Transepithelial Electrical Resistance Measurements

HT-29 and HT29-RevMTX cells (first passage in drug-free medium) were seeded at 2 × 10^5 cells per well on tissue culture-treated Transwell polycarbonate membranes, 24.5-mm dia, 0.4-μm pore size (Costar, Cambridge, MA), modified in the laboratory for the passage of electrodes. Culture conditions were as described above. Electrical measurements were performed after 30 d in culture, under sterile conditions, using the same medium, but supplemented with 10 mM Hepes and 5% FBS only. Measurements were performed using Ag/AgCl microelectrodes (Millipore Corporation, Bedford, MA) connected to a VCL 600 voltage-current clamp instrument (Physiological Instruments, Houston, Texas). Values were measured after 1-s current pulses (5 μA) and corrected for the resistance of empty filters.

Table I. Activities of Brush Border–associated Hydrolases in Parental and MTX-adapted HT-29 Cells

		Cells adapted to MTX		
	Parental cells	10^{-5} M	10^{-4} M	10^{-3} M
DPP-IV	20 ± 0.5	111 ± 2	121 ± 0.5	128 ± 2
Sucrase	nd*	nd	nd	nd

Enzyme activities were measured in the cell homogenates of late postconfluent cultures (day 28 after seeding). Each value is the mean of four to five determinations made at different passages. Results are expressed as milliunits/mg protein. * nd, not detectable. Similar values were observed in corresponding HT29-RevMTX cells (not shown).

Comparisons were made with the unpaired Student's test with $P < 0.01$ as the significance level.

Dihydrofolate Reductase Binding and Activity Assays

Assays were performed on exponentially growing cells (5 d after seeding). Cells adapted to the different concentrations of MTX were used at the first passage in the absence of drug in order to avoid competition with MTX. Cells were detached with trypsin and EDTA, washed three times in PBS Ca^2Mg^2-free, and stored in liquid nitrogen for further studies. The cell pellets were extracted in Tris HCl buffer 50 mM, pH 7.5, sucrose 0.25 M. The assays were performed on 105,000 *g* supernatants which were stored as aliquots at −80°C. The protein content was estimated using a Bio-Rad kit (Bio-Rad, Richmond, CA) with albumin as standard. Two different assays were run in parallel, one measuring MTX binding on DHFR, and the other the enzyme activity. DHFR binding assay was performed according to the method described by Kamen et al. (1983). The final composition of the assay mixture was phosphate buffer 50 mM, pH 6, NADPH 0.05 mM, KCl 25 mM, beta-mercaptoethanol 15 mM, albumin 5 mg/ml, [^{3}H]methotrexate 0.05 μCi (30 Ci/mmol) and sample to measure, purified DHFR (Sigma Chemical Co.) or newborn rat liver extract. After 5 min of incubation at 30°C, the reaction was stopped by adding 50 μl of a charcoal suspension (charcoal 5 mg/ml, dextran 0.5 mg/ml, albumin 2.5 mg/ml, EDTA 50 mM). The tubes were then centrifuged, an aliquot of the supernatant mixed with Instagel (Packard Instrument Co., Downers Grove, IL), and the radioactivity measured by liquid scintillation counting. Linearity was verified with purified DHFR and newborn rat liver extract. The results are expressed as mol labeled MTX bound/mg protein. DHFR activity was estimated by measuring the decrease in absorbance at 340 nm, according to a method adapted from Bertino et al. (1964). The composition of the assay mixture was: Tris HCl 100 mM, pH 7.5, beta-mercaptoethanol 10 mM, KCl 100 mM, NADPH 0.1 mM, dihydrofolate 0.1 mM, and cell extract. The assay was performed at 30°C. Linearity with protein content was verified and rat liver extract was run each time in parallel as internal standard. The results are expressed as nmol/min/mg protein.

Southern Blot Analysis

DNA was prepared from postconfluent cells (day 28) using proteinase K and RNase A (Boehringer Mannheim Biochemicals) and phenol, as described (Davis et al., 1986). For Southern blotting (Southern, 1975), DNAs (8 μg/sample) were digested with restriction nuclease EcoRI (Boehringer Mannheim Biochemicals), electrophoresed on 0.8% agarose gel in Tris borate EDTA buffer (Sambrook et al., 1989), and transferred to Hybond N (Amersham Corp., Amersham, UK). Filters were prehybridized (3 h, 65°C) in a solution containing 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 20 μg salmon sperm DNA per ml. A human DHFR-genomic DNA insert (ATCC CHB 203) (Anagnou et al., 1984; Chen et al., 1982) was obtained from the American Type Culture Collection (Rockville, MD), and labeled with ^{32}P using multiprime DNA labeling system (Amersham Corp.). Hybridization with the ^{32}P-labeled probe was done overnight at 65°C in the same solution as prehybridization. Before autoradiography, blots were washed at 65°C, twice in 2 × SSC, once in 2 × SSC, 0.1% SDS, and then once in 0.1 × SSC. To normalize for DNA, filters were dehybridized and rehybridized with a cDNA clone for human sucrase-isomaltase, SI_2 (Green et al., 1987), a gift from Dr. D. Swallow (Medical Research Council, University College, London). Quantitation was achieved by scanning the bands of fluorograms, using a densitometric scanner (model Mark III CS; Joyce, Loebl and Co., Ltd., Gatehead, UK).

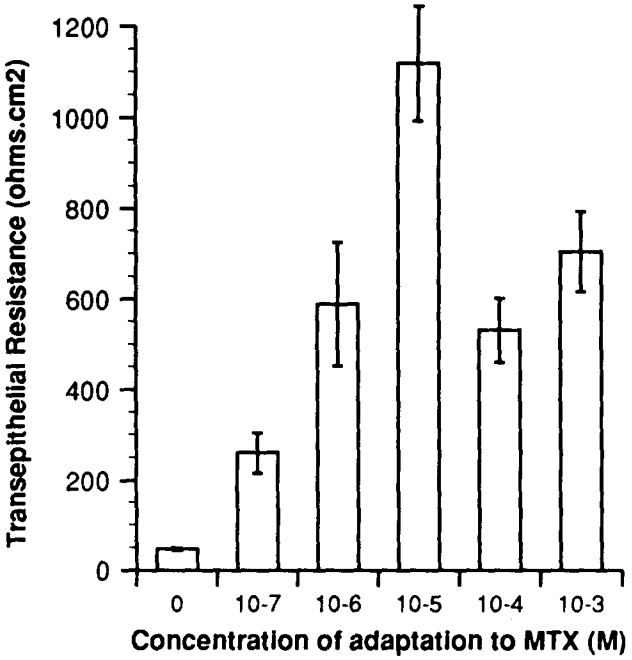

Figure 7. Transepithelial resistance (R_T) of control and MTX-adapted HT-29 cell cultures grown on filters. Results from each population are the mean and SD of measurements of six filters made at 0, 2, 12, 24, 48, and 72 h. Values for each filter were found to be stable from one measurement to another. R_T from all MTX-adapted populations were significantly different from control cells ($P < 0.001$) with the values from HT29-MTX10^{-5} being significantly different from those from the other MTX-adapted cells ($P < 0.001$).

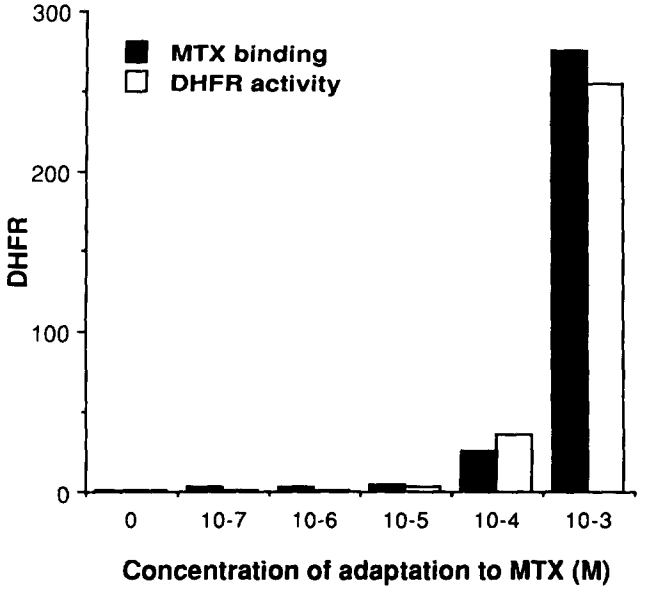

Figure 8. Dihydrofolate reductase measurements in parental and MTX-adapted HT-29 cells. Results of MTX binding assays are expressed as 10^{-4} mol MTX bound per mg of protein. Precise values corresponding to histograms are 1.29 ± 0.06, 3.29 ± 0.2, 3.06 ± 0.06, 4.7 ± 0.25, 25.4 ± 0.8, and 276.3 ± 8.5 for parental and cells adapted to 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M MTX, respectively. Results of enzyme activities are expressed as nmol/min/mg of protein. Precise values corresponding to histograms are 0.673 ± 0.163, 1.61 ± 0.063, 1.726 ± 0.054, 3.546 ± 0.146, 36.22 ± 1.054, and 256.0 ± 7.41 for parental and cells adapted to 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M MTX, respectively.

Results

Adaptation of HT29-MTX10^{-5} to 10^{-4} and 10^{-3} M MTX

The kinetics of adaptation of HT29-MTX10^{-5} cells to 10^{-4} and 10^{-3} M MTX is shown in Fig. 1. For each concentration there is a high rate of mortality in the first days of the first passage, followed by a slow resumption of cell growth. The growth rate then increases progressively in the following subcultures, until stabilization of the growth curve after several passages (7 and 10 for cells adapted to 10^{-4} and 10^{-3} M MTX, respectively). This pattern of adaptation resembles that seen in the adaptation to lower concentrations of the drug (Lesuffleur et al., 1990). IC_{50} of HT29-MTX 10^{-4} and HT29-MTX 10^{-3}, and comparison with the values in HT-29 cells and in subpopulations adapted to lower concentrations, is reported in Fig. 2.

Dose-dependent Modification of the Differentiated Phenotype

Analysis of the differentiation pattern of HT-29 subpopulations stably adapted to 10^{-4} and 10^{-3} M MTX (HT29-MTX10^{-4} and HT29-MTX10^{-3}) shows a clear-cut modification of the differentiated phenotype, compared with HT29-MTX10^{-6} and 10^{-5} cells.

Postconfluent cultures of HT29-MTX10^{-6} and 10^{-5} cells, and of the same cells reversed to drug-free medium, form a monolayer of polarized goblet cells. These goblet cells all secrete, from the tenth to fourteenth day on, large amounts of mucins which are positively stained with PAS and alcian blue (Fig. 3, *c* and *d*) and immunoreact with antibodies against gastric, but not colonic, mucins (Fig. 4 *g*). They also exhibit a discrete apical brush border (Fig. 5 *a*) and show an apical expression of villin, DPP-IV, and CEA (Fig. 4, *a*, *c*, and *e*). In contrast, late postconfluent cultures of HT29-MTX 10^{-4} and 10^{-3} cells are no longer of the gobletlike type, but display an absorptive-like pattern of differentiation. They form a monolayer of polarized cells which form domes (Fig. 6). The majority of these cells show no sign of mucus secretion (Fig. 3, *e* and *f*; Fig. 4 *h*; and Fig. 5 *b*), except for a very few cells (<0.01%) which show some discrete mucus secretions at immunofluorescence (Fig. 4 *h*) and transmission EM (Fig. 5 *b*). These aborptive-like cells, like the cells adapted to lower concentrations (10^{-6} and 10^{-5} M), exhibit an apical brush border (Fig. 5, *b* and *c*) which expresses villin (Fig. 4 *b*) and DPP-IV (Fig. 4 *d*; Table I) and have an elevated transepithelial resistance (Fig. 7). However CEA is not expressed (Fig. 4 *f*). This differentiation pattern is stable, as it is observed in all the subsequent passages (up to 15–30) of the two subpopulations and maintained when the cells are subcultured in drug-free medium (up to 25 passages). Like in populations adapted to lower concentrations of MTX (10^{-7} to 10^{-5} M) no expression of SI could be detected, whether by immunofluorescence (not shown) or enzyme activity assays (Table I). This absence of SI is consistent with the observation that the expression of the enzyme is repressed when HT-29 cells are grown, as in this case, in the presence of glucose (Huet et al., 1987; Zweibaum et al., 1985). No *P*-glycoprotein could be detected either (not shown).

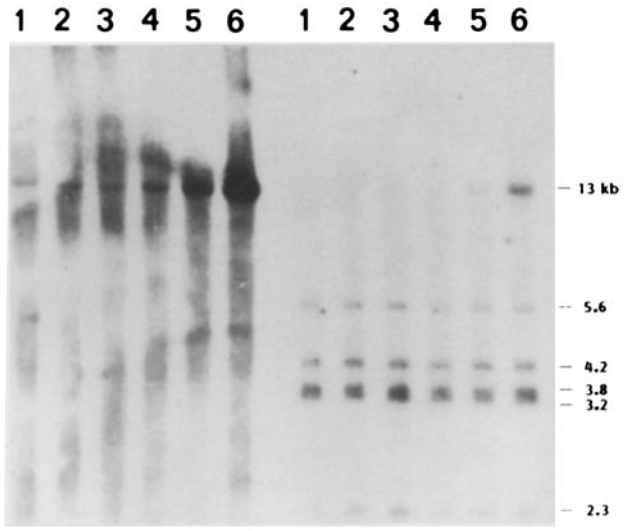

Figure 9. Southern blot analysis of DNA from parental and MTX-adapted HT-29 subpopulations. DNA was extracted from post-confluent cultures of cells stably adapted to the corresponding concentrations of MTX. Numbers correspond to parental cells (*1*) and to cells adapted to MTX 10^{-7} M (*2*), 10^{-6} M (*3*), 10^{-5} M (*4*), 10^{-4} M (*5*), and 10^{-3} M (*6*). (*Left*) Hybridization with probe CHB 203 for human DHFR (see Materials and Methods). (*Right*) The same filter was dehybridized and rehybridized, as control, with probe SI_2 for human sucrase-isomaltase. Note the increased signals for the 13-kb band of DHFR in lanes *5* and *6* (*left*) which could not be completely dehybridized as shown in lanes *5* and *6* (*right*). Coefficients of amplification of the 13-kb band of DHFR, as deduced from scanning analysis, were 1.1, 1.3, 2.9, 17.4, and 52.7 for cells adapted to 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M MTX, respectively.

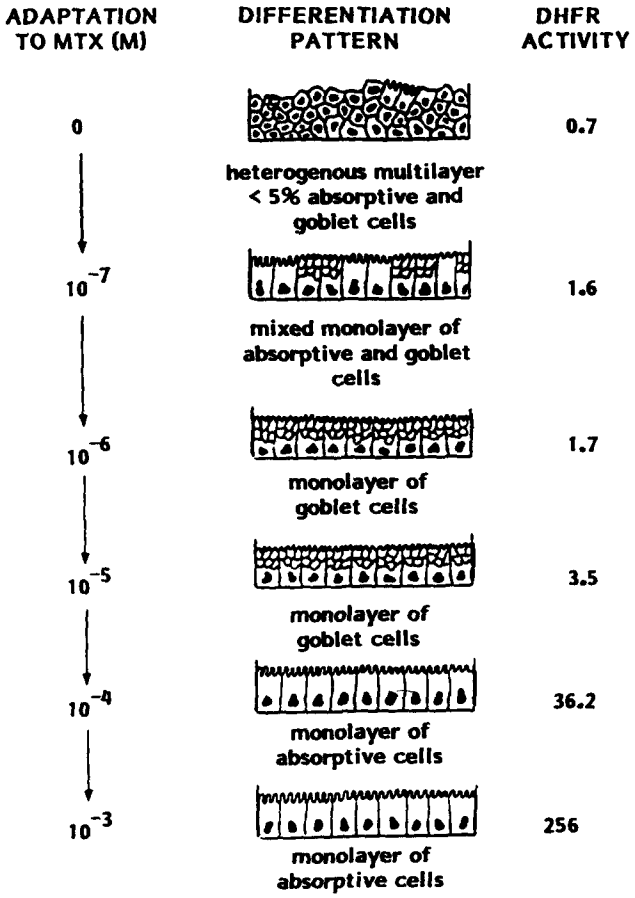

Figure 10. Schematic representation of the relationship differentiation pattern/DHFR activity in HT-29 cells stably adapted to increasing concentrations of MTX. For the differentiation pattern of parental, HT29-MTX10^{-7}, and HT29-MTX10^{-6} cells (see Lesuffleur et al., 1990). DHFR activity refers to the data in Fig. 8.

Dose-dependent Amplification of the DHFR Gene

The intracellular content of DHFR increases only slightly with the first concentrations of adaptation to MTX (10^{-7} to 10^{-5} M), but shows a dramatic increase at the upper concentrations of adaptation, reaching values which are 20 and 214 times (binding assay) and 54 and 380 times (enzyme activity) higher than in parental cells in HT29-MTX10^{-4} and HT29-MTX10^{-3} cells, respectively (Fig. 8). This increase of the enzyme content and activity parallels an amplification of the DHFR gene as substantiated by Southern blot analysis (Fig. 9). The relationship between the dose-dependent levels of DHFR and the differentiation pattern of the corresponding cultures is summarized in Fig. 10.

Discussion

We report here for the first time a shift in the pattern of differentiation of HT-29 cells from one differentiated phenotype to another, i.e., from mucus-secreting to absorptive type cells. This change in phenotype occurs in the course of adaptation of the cells to increasing concentrations of MTX. It is concomitant with a large amplification of the DHFR gene. Amplification of this gene is a well-known mechanism of acquired resistance to MTX (Schimke, 1984). Its occurrence, like in the present case, is often associated with resistance to very high drug concentrations (Srimatkandada et al., 1983, 1989).

The reasons for the change in phenotype are unclear. A clonal selection of a hypothetical population of absorptive cells present in cultures adapted to 10^{-6} and 10^{-5} M MTX is unlikely, since one would have expected to detect populations of these cells in the course of the numerous passages maintained at these concentrations of MTX (at least 50). It is also most unlikely that the shift of differentiation is a direct consequence of MTX "per se," as it is maintained when the cells are subcultured in drug-free medium. However there may be a causal relationship between the amplification of the DHFR gene and the change in phenotype. DHFR is a key enzyme in the folate cycle (Morrison and Allegra, 1989) and the increased activity of the enzyme may result in an impairment of the normal balance of intermediate metabolites and cofactors associated with this cycle. Accordingly, the disappearance of mucins simply could be a result of a decreased availability of one of the substrates of the folate cycle, namely serine, which is a major aminoacid in the peptide core of mucins (Neutra and Forstner, 1987). Another possibility is that increased DHFR activity results in modifications of the pool of 5-methyltetrahydrofolates. This might result in a change of availability of methyl groups and therefore alterations of DNA and RNA methylation which, in turn, may lead to changes in differentiation (Bird, 1986; Jones, 1986). Such a mechanism could interfere with the normal expression of genes coding for mucins and CEA, either directly, or in-

directly through modifications of expression of the enzymes involved in their glycosylation. Lastly, it cannot be excluded that the phenotypic change observed is independent from DHFR amplification, but results from other genetic changes associated with drug-induced chromosomal modifications (Bostock et al., 1979), as shown in HT-29 cells adapted to 5-fluorouracil (Lesuffleur et al., 1991).

Whatever the mechanisms, it is of interest to note that the shift in differentiation is reminiscent of the late developmental stages of the human colon; indeed, the midgestational colonic luminal epithelium is exclusively formed of polarized goblet cells which, like in HT29-MTX10^{-6} and 10^{-5} cells, show an apical expression of villin and brush-border hydrolases, whereas, in the late stages of gestation, this epithelium is exclusively formed of absorptive cells (Kornowski, A., T. Lesuffleur, Y. Kedinger, A. Salmon, and A. Zweibaum, manuscript in preparation). Interestingly, HT29-MTX mucus cells at low drug concentration have a transepithelial resistance, a characteristic which has been shown to be associated with columnar absorptive cells, like Caco-2 cells (Grasset et al., 1984) and the transporting clone HT29-19A (Augeron et al., 1986). Electrical resistance has not, to our knowledge, been studied before in cultures of mucus colonic cells (Augeron and Laboisse, 1984; Huet et al., 1987). Whether midgestational colonic mucus cells also display functional characteristics of absorptive cells is unknown.

Further studies on these HT-29 cells and on the mechanisms responsible for the modifications observed should be helpful in unraveling the factors which control the commitment of normal intestinal cells to the alternative types of differentiation, namely goblet or absorptive cells.

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