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Growth Adaptation to Methotrexate of HT-29 Human Colon Carcinoma Cells Is Associated with Their Ability to Differentiate into Columnar Absorptive and Mucus-secreting Cells[1]

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ABSTRACT

The purpose of this work was to investigate whether the phenomenon of metabolic adaptation of HT-29 cells to glucose deprivation and subsequent emergence of differentiated subpopulations (A. Zweibaum *et al.*, J. Cell. Physiol., *122:* 21–29, 1985) also applies to anticancer drugs that act at a metabolic level like methotrexate (MTX). Stepwise adaptation of exponentially growing HT-29 cells to increasing concentrations of MTX (10^{-7}, 10^{-6}, and 10^{-5} mol) results, after a phase of high mortality, in the emergence of subpopulations with stable growth rates and curves close to those of untreated control cells. In contrast to control cells which are heterogenous and contain, after confluency, only a small proportion of differentiated cell types (less than 4%), postconfluent cultures of MTX-adapted cells are totally differentiated. Cells adapted to 10^{-7} M MTX form a mixed population of columnar absorptive and mucus cells; at higher concentrations cells are almost exclusively of the mucus-secreting type. All cells, whether mucus-secreting or not, develop an apical brush border which strongly expresses dipeptidylpeptidase IV, carcinoembryonic antigen, and villin. These differentiation features, which resemble those of fetal colon, are associated with decreased rates of glucose consumption and lactic acid production. Both differentiation characteristics and metabolic changes are stably maintained when the cells are subcultured in the absence of the drug. Like the original population, MTX-adapted cells are tumorigenic in nude mice. We propose that cells which are able to differentiate and which are the origin of the small proportion of differentiated cell types found in postconfluent cultures of the original cell line possess an advantage which allows them to be adaptable to "metabolic stress" conditions.

INTRODUCTION

Human colon carcinoma contains a variable proportion of cells that exhibit the ultrastructural morphology of either columnar absorptive (1–4) or mucus-secreting cells (3, 5). These morphological characteristics of differentiation are associated with the presence of structural and functional proteins that are found in the normal or fetal gastrointestinal tract epithelium. Mucous cells have been shown to secrete mucins of normal colonic (6–8) and also, as in the fetal colon, gastric immunoreactivity (7, 8). Columnar absorptive cells express, in addition to CEA[3] (9, 10) and villin (11), hydrolases normally associated with the small intestine (12) and fetal colon (13, 14), such as sucrase-isomaltase, aminopeptidase N, dipeptidylpeptidase IV, and alkaline phosphatase (14).

Whether the presence of these differentiated phenotypes with morphological and functional characteristics close to those of normal or fetal colonic epithelial cells is simply one more example of tumor heterogeneity or if it has a particular significance as to the prognosis of these cancers or the poor results of chemotherapy is a question that, until recently, could not be addressed satisfactorily. Indeed, the recent development of specific antibodies against differentiation-associated proteins, such as mucins from normal colon (15) or stomach (8), villin (16), or brush border hydrolases (17), now allows rapid and sensitive screening of pathological material. Moreover, recent reports have shown that some colon carcinoma cell lines are able to express in culture the differentiation characteristics of either columnar absorptive (18–21) or mucus-secreting cells (21–23). Such cell lines provide an important model for further investigation of the biological significance, and possible adaptation advantages, of differentiated colon cancer cell types.

Among these cell lines, HT-29 (24) appears as the most appropriate for such studies. Indeed, HT-29 tumors in nude mice contain both mucous (6) and columnar absorptive cells (14, 25). HT-29 cells in culture, although essentially undifferentiated (18, 20–22, 26, 27), are also heterogenous in that they contain a small proportion of mucous cells (15, 22) and columnar absorptive cells organized around lumina (18), both types of cells having not been characterized as yet as to the proteins they express. Even more importantly, HT-29 cells grown in various metabolic stress conditions, such as glucose deprivation (18, 21, 26), glutamine deprivation (28), or sodium butyrate treatment (22, 29), are able, after an initial phase of mortality, to undergo growth adaptation to these conditions. This growth adaptation is followed by the emergence of differentiated cell populations of either absorptive (18, 21, 26, 28, 29) or mucus-secreting cells (21, 22). Although the mechanisms involved in metabolic adaptation and differentiation are still unknown, it can be postulated that these differentiated populations evolve from this small population of cells that are present in the original cell line and are able to differentiate.

Whether this adaptability to stress conditions is a general metabolic property of those carcinoma cells having the ability to differentiate led us to investigate if anticancer drugs that act at a metabolic level had a similar effect on growth and differentiation of HT-29 cells. We report here the results obtained with MTX, a drug that interferes with a metabolic pathway, namely that of folates, by inhibiting the enzyme DHFR (30).

MATERIALS AND METHODS

Cell Culture. HT-29 cells were obtained from Dr. Jorgen Fogh (Sloan Kettering Memorial Cancer Center, Rye, NY) and were used between passages 144 and 200 of the cell line. Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France), supplemented with 10% inactivated (30 min, 56°C) fetal bovine serum (Boehringer, Mannheim, Germany). All experiments and maintenance of the cells were done in 25-cm² T-flasks (Corning Glassworks, Corning, NY) at 37°C in a 10% CO_2/90% air

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; MTX, methotrexate; PBS⁻, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; DPP-IV, dipeptidylpeptidase IV; IC_{50}, concentration of drug that causes a 50% inhibition of control cell growth; DHFR, dihydrofolate reductase.

Table 1 *IC_{50} of MTX in control and MTX-adapted HT-29 cells*

IC_{50} was measured after 5 days in culture in the presence of the drug (see "Materials and Methods").

	Control cells	Cells adapted to MTX		
		10^{-7} mol	10^{-6} mol	10^{-5} mol
IC_{50}	$3.3 \pm 0.5 \times 10^{-8}$ M[a]	1.65×10^{-7} M (P11)[b] 3.20×10^{-7} M (P17) 2.70×10^{-7} M (P24)	1.1×10^{-5} M (P14) 1.0×10^{-5} M (P21)	2×10^{-5} M (P8) 2.6×10^{-5} M (P12)

[a] Mean ± SD of 15 determinations made between passages 144 and 200.
[b] Single determinations made at the indicated passages (P).

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^-. 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. Cell growth was assessed after 5 days by using a methylene blue technique as described (31). This long exposure time was chosen on the basis that differentiation is a growth-related event which takes place after the cells have reached confluency (18–22, 26, 28, 29). It was therefore necessary to determine a concentration at which a sufficient proportion of the cells would survive in long-term experiments. MTX was from Sigma (St. Louis, MO).

Indirect Immunofluorescence and Antibodies. Indirect immunofluorescence was performed concomitantly on unpermeabilized cell layers and on frozen cryostat sections (6 μm thick). The same cultures, grown in T-flasks, were used for both assays. Two thirds of the cell layer were detached with a rubber policeman, rolled up, snap frozen in liquid nitrogen, and used for cryostat sections. The remaining one third was used for surface immunofluorescence. Both types of preparations were fixed for 15 min at room temperature in 3.5% paraformaldehyde in PBS^-. Mouse monoclonal antibody HBB 3/775/42, specific for human intestinal DPP-IV (17), was obtained from Dr. Hans-Peter Hauri (Biocenter of the University of Basel, Basel, Switzerland). Polyclonal rabbit antibodies against sucrase-isomaltase purified from Caco-2 cells were produced in the laboratory (27). Rabbit polyclonal antibodies against porcine villin (16) were a gift from Dr. Daniel Louvard (Institut Pasteur, Paris, France). Monoclonal antibody 601 against colonic CEA was obtained from Biosys (Compiègne, France). Polyclonal antibodies against individual colonic mucins were produced in rats and rabbits immunized with semipurified, water-soluble, heated extracts from 30 samples of normal human colonic mucosa (32, 33). Among 2 groups of 30 immune sera, 2 were selected, one from a group of 5 rats and another from one rabbit that was immunized with colonic extracts from the same blood group O donor. Gastric mucosa extracts from this donor were similarly used for obtention of a rabbit immune serum against gastric mucins. All rabbits used for the production of immune sera were of the A^+ phenotype (34) in order to avoid the presence of anti-blood group A antibodies. Immune sera against colonic and gastric mucosa extracts were further absorbed with gastric mucosa and colonic mucosa dry acetone powders (32), respectively, to eliminate non-organ-specific antibodies. Specificity of absorbed immune serum was checked by immunofluorescence on cryostat sections of a panel of 30 normal adult colons and stomachs. They were also checked on human red blood cells for the absence of anti-ABH blood group activity. Antibodies against blood group A antigen were produced in A^- rabbits (34) immunized i.v. with human blood group A red cells. Monoclonal antibody JSB-1 (Sanbio, Uden, Netherlands) and MRK-16 (35), kindly provided by T. Tsuruo (Cancer Chemotherapy Center, Tokyo, Japan) were used for the detection of the P-glycoprotein. Anti-rabbit and anti-rat fluorescein-coupled sheep antiglobulins were from Institut Pasteur Productions (Paris, France). Anti-rabbit rhodamine-coupled sheep antiglobulins were from Silenius Laboratories (Hawthorn, Australia). Anti-mouse fluorescein-coupled rabbit antiglobulins were from Cappel Laboratories (Cochranville, PA).

Electron Microscopy. Transmission electron microscopy was performed on cells grown in 25-cm^2 plastic flasks as previously reported (18, 19). Samples embedded in Epon were reembedded in order to make sections perpendicular to the bottom of the flask.

Enzyme and Biochemical Assays. Activities of sucrase (EC 3.3.1.48), dipeptidylpeptidase IV (EC 3.4.14.5), aminopeptidase N (EC 3.4.11.2), and alkaline phosphatase (EC 3.1.3.1) were measured in the cell homogenate and in a brush border-enriched fraction as previously reported (20). Enzyme activities are expressed as milliunits per mg of protein. One unit is defined as the activity which hydrolyzes 1 μmol of substrate per min at 37°C. Proteins were measured by the method of Lowry *et al.* (36). Glucose consumption, lactic acid production, and glycogen content were measured as reported (37).

Tumors in Nude Mice. Tumors were produced in nude mice by s.c. injection as reported (14, 25, 38).

RESULTS

Growth Adaptation to MTX. Based on an IC_{50} of 3.0×10^{-8} mol (Table 1), cells were first adapted to grow in the presence of 1×10^{-7} M MTX. Control untreated cells (Fig. 1*A*) have a doubling time of 24 h, reach confluency after 6 days, and are stationary after 10 days. By comparison, cells seeded in the presence of 10^{-7} M MTX have a slow rate of growth in the first 48 h and then show an important mortality. This phenomenon stops after 12 days, after which cell growth resumes at a very low rate (Fig. 1*A*). After 30 days, cells are not confluent but organized into clusters which represent only 5% of the control and do not reach confluency until 3 mo. As shown in Fig. 1*B*, subsequent weekly passage of surviving MTX-treated cells results, after a few passages, in a stabilization of the growth curve. Stepwise adaptation to increasing concentrations shows the same pattern of cell mortality and stabilization of the growth curves (Fig. 1, *C* to *F*). The IC_{50} of cells adapted to 10^{-7}, 10^{-6}, and 10^{-5} M MTX was 7, 300, and 700 times higher than in control cells, respectively (Table 1).

Differentiation Characteristics of Control and MTX-adapted Cells. With regard to previous observations which showed that differentiation, when it occurs, is complete after late confluency (18–22, 26, 28, 29), all cultures were examined 30 days after seeding.

Control Cells. As shown in Figs. 2 and 3, the cell layer of postconfluent HT-29 cells, although essentially constituted of undifferentiated cells, contains a small proportion of columnar absorptive and mucous cells. Columnar absorptive cells express DPP-IV and CEA (but not sucrase-isomaltase) and are of two types. Some are organized, as previously reported (18), around intracellular lumina (Figs. 2*b* and 3*c*); others, which had not been detected before, are organized into small clusters with an apical brush border directed toward the culture medium (Figs. 2*a* and 3*b*). Mucous cells (Fig. 3*a*) are found to be of two types according to the colonic or gastric immunoreactivity of the mucins they secrete (Fig. 2, *e* to *h*). Altogether the proportion of differentiated cell types can be estimated as less than 4%.

MTX-adapted Cells. The totality of postconfluent MTX-adapted cells is differentiated, with a proportion of mucus-

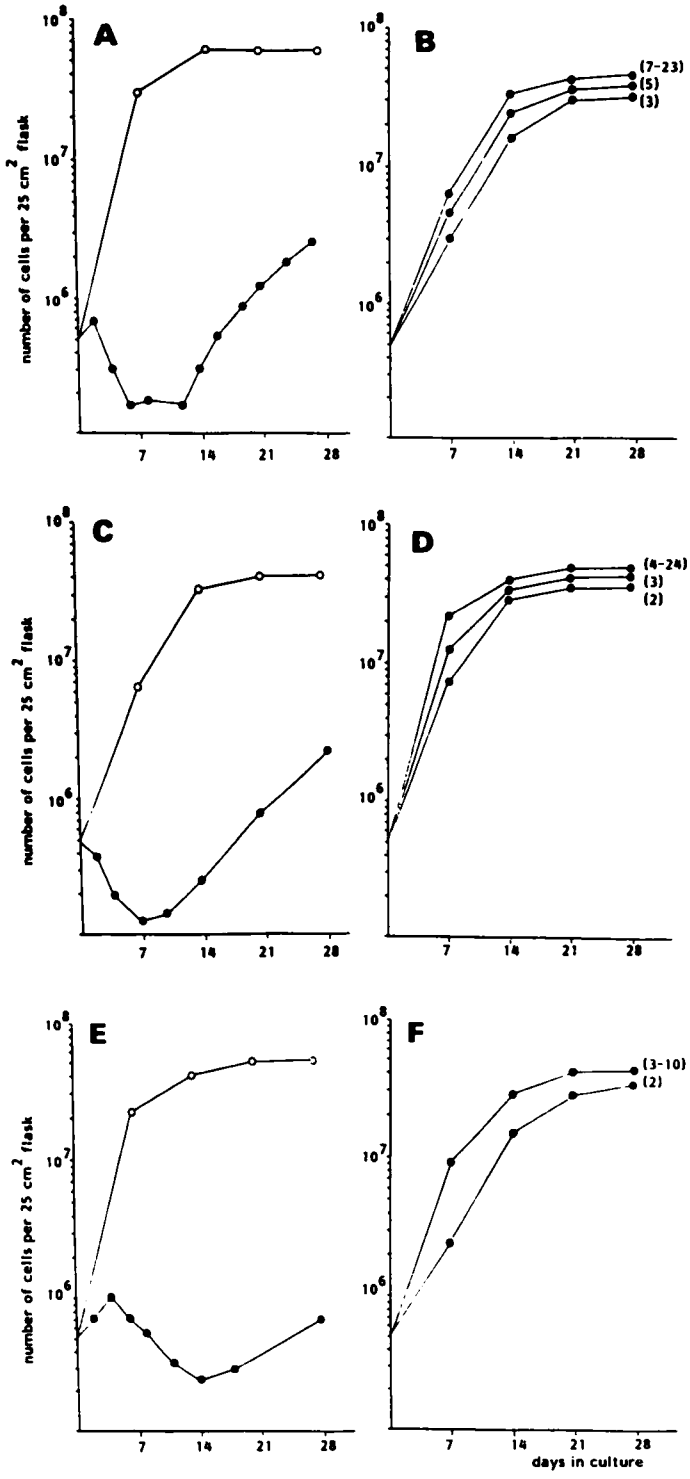

Fig. 1. Growth adaptation to methotrexate by HT-29 cells. *A* and *B*, adaptation to 10^{-7} M MTX; *A*, growth curves of control cells (○) and of the same cells cultured in the presence of 10^{-7} M MTX (●); *B*, growth curves of subsequent weekly passages in the presence of 10^{-7} M MTX. *Numbers in parentheses*, the number of passages with passage 2 corresponding to MTX-treated cells (*A*) harvested after 28 days of treatment. *C* and *D*, adaptation of 10^{-7} M MTX-adapted cells to 10^{-6} M MTX. *C*, growth curves of 10^{-7} M MTX (passage 16) (○) and of the same cells cultured in the presence of 10^{-6} M MTX (●); *D*, subsequent weekly passages in the presence of 10^{-6} M MTX of drug-treated cells (*C*), harvested after 28 days. *E* and *F*, adaptation of 10^{-6} M MTX-adapted cells to 10^{-5} M MTX. *E*, growth curves of 10^{-6} M MTX-adapted cells (passage 6) (○) and of the same cells cultured in the presence of 10^{-5} M MTX (●); *F*, subsequent weekly passages in the presence of 10^{-5} M MTX of drug-treated cells (*E*) harvested after 28 days.

secreting cells which increases with the concentration of the drug to which the cells are adapted. In 10^{-7} M MTX-adapted cells, half of the cells are of the columnar absorptive cell type, and the other half are of the mucus-secreting type (Fig. 4). Although a majority of mucins show a gastric immunoreactivity (Fig. 5, *a* and *c*), a proportion of secretions, much higher than in control cells, reacts with anticolonic mucin antibodies (Fig. 5*e*). At higher adaptation concentrations (10^{-6} and 10^{-5} mol), almost the totality of the cells are of the mucus-secreting type (Fig. 4*b*) with secretions expressing almost exclusively a gastric immunoreactivity (Fig. 5, *b*, *d*, and *f*). As shown in Fig. 5, *a* and *b*, mucus secretions are excreted at the cell surface. Whatever the concentration of drug the cells are adapted to, all cells show an apical expression of DPP-IV, CEA, and villin whether or not they secrete mucins (Fig. 6). As shown in Table 2 the enzymatic differentiation of MTX-adapted cells is restricted to DPP-IV. These differentiation characteristics were found to be very stable from passage to passage. Their occurrence is growth related as disclosed by immunofluorescence which shows that the first immunoreactive secretions as well as the first apical immunoreactivity appear after 8 to 10 days in culture and increase progressively afterward (Fig. 6, *a* and *b*); this is quantitatively supported by the growth-related increase of DPP-IV activities (Fig. 7). In none of the MTX-adapted cell populations was the P-glycoprotein detected. No blood group A immunoreactivity was associated with mucus secretion.

Irreversibility of the Differentiation of MTX-adapted Cells. After stabilization of the growth curve, cells adapted to 10^{-7} M MTX (passage 8) and 10^{-6} M MTX (passage 6) were reverted to drug-free medium and subsequently cultured for several passages in the absence of the drug (34 and 21 passages for cells originating from 10^{-7} and 10^{-6} M MTX-adapted cells, respectively). The growth curve was unchanged as compared with the corresponding MTX-treated cells (not shown). The differentiation characteristics, as assayed at every other passage by immunofluorescence, or after 10 and 20 passages by transmission electron microscopy, were strictly the same as in MTX-treated cells (not shown). The stability of the differentiation is quantitatively supported by the results of DPP-IV activity assays (Table 2).

Glucose Metabolism Characteristics of Differentiated MTX-adapted and Reverted Cells. As shown in Table 3, the rates of glucose consumption and lactic acid production decrease with MTX concentration increments. Glycogen content is increased in a parallel manner, although slightly. These values are independent from the presence of MTX as they are the same in the corresponding MTX-adapted cells grown in the absence of the drug (Table 3).

Tumors in Nude Mice. Nude mice given injections of MTX-adapted cells developed tumors which had apparently the same tumor growth rate as that of tumors developed with control cells. As compared with the control tumors, which are poorly differentiated, xenografts from MTX-adapted cells are well differentiated and strongly express both mucus and DPP-IV (not shown).

Reversibility of IC_{50}. The IC_{50} of cells that reverted from 10^{-7} and 10^{-6} M MTX was measured regularly at different passages. Only cells that reverted from 10^{-7} M MTX were cultured for a sufficient number of passages to observe a modification of IC_{50} with values decreasing to 1.8×10^{-7}, 7×10^{-8}, and 6×10^{-8} mol at passages 10, 24, and 34, respectively.

Absence of Effect on Differentiation of Treatment of Post-confluent Cells. Postconfluent HT-29 cells (Day 14) were

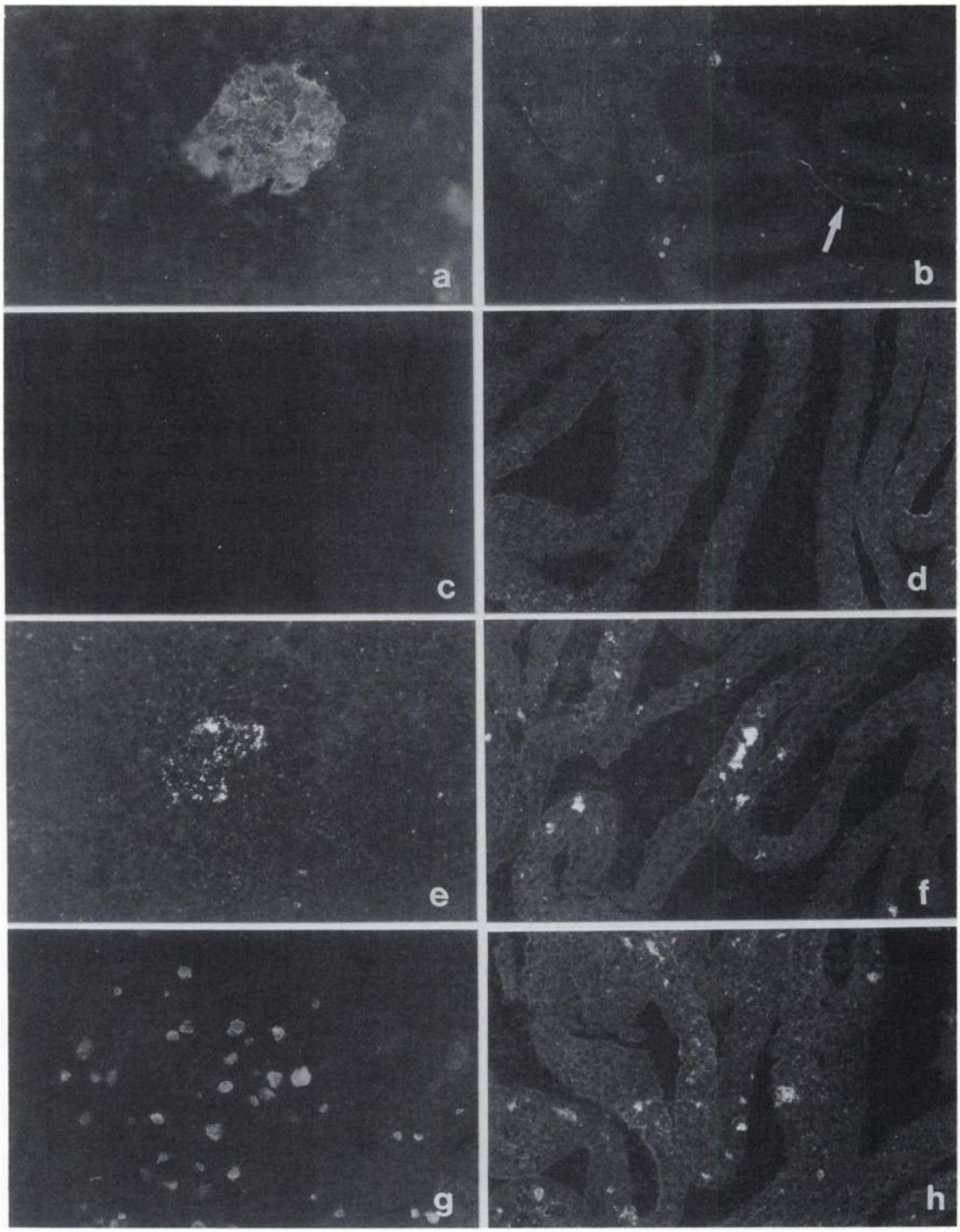

Fig. 2. Indirect immunofluorescence staining of differentiation-associated proteins in late postconfluent cultures (Day 30) of HT-29 cells. *a*, surface labeling of DPP-IV showing a cluster of cells with an apical expression of DPP-IV. The total surface of DPP-IV-immunoreactive clusters, measured in 5 different cultures, equals 2% of total cell layer surface. *b*, expression of DPP-IV in cryostat sections showing a zone of apical staining (*arrow*) and the presence of several positive lumina. *c*, absence of immunodetectable sucrase-isomaltase; *d*, villin, cryostat section showing a faint staining of several lumina; *e*, expression of gastric specific immunoreactive secretions on the cell surface and, for *f*, in cryostat sections; *g*, expression of colonic specific immunoreactive secretions on the cell surface and in cryostat sections (*h*). Secretions which react with anti-colonic mucin antibodies are different from those reacting with anti-gastric mucin antibodies as disclosed by double labeling experiments (not shown). Expression of CEA (not shown) was the same as that of DPP-IV. No expression of the P-glycoprotein could be detected. The same results were obtained in 20 different cultures between passages 144 and 200. × 85.

treated for 50 days with increasing concentrations of MTX, ranging from 10^{-7} to 10^{-5} mol. The effect on cell mortality was dose dependent (Fig. 8*A*). The remaining cells were harvested and seeded back in the absence of the drug. Growth curves were close to those of control cells (Fig. 8*B*). After 30 days, cell cultures were analyzed by immunofluorescence for their differentiation characteristics. Whatever the concentration of MTX the cells were rescued from, they exhibited the same small

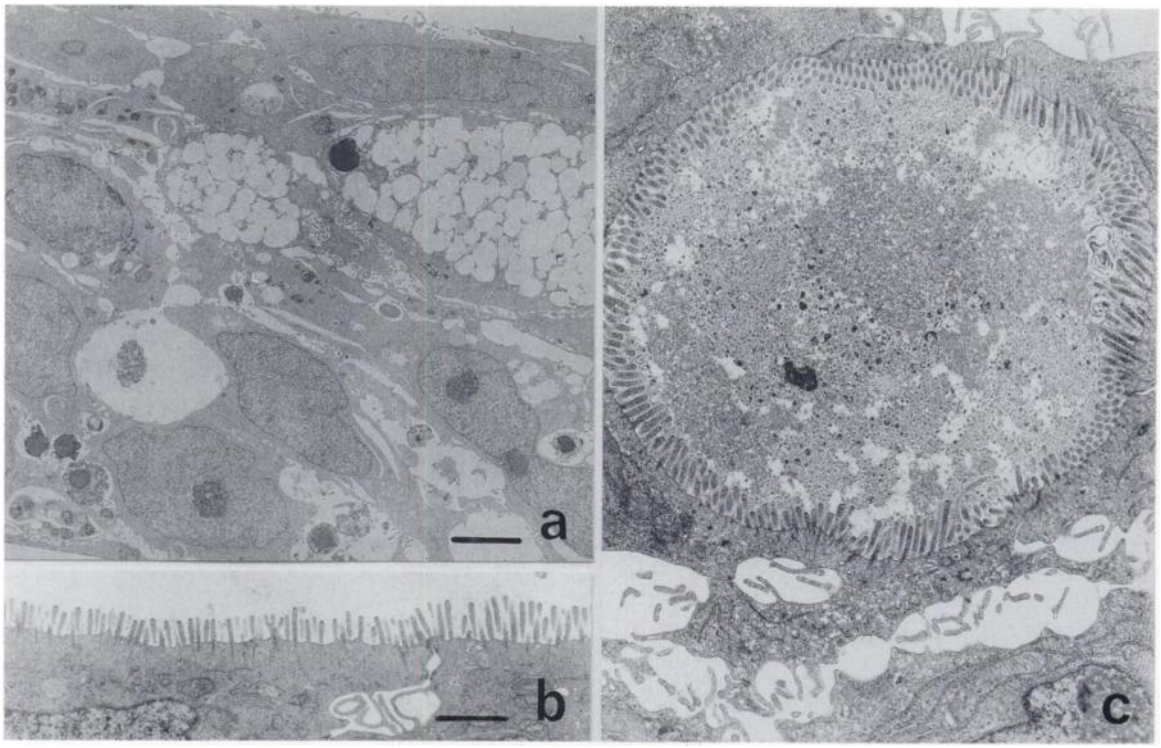

Fig. 3. Transmission electron microscopy of postconfluent cultures (Day 30) of HT-29 cells. Sections are perpendicular to the bottom of the flask. *a*, presence of a goblet cell embedded into the cell multilayer (*bar*, 6 μm); *b*, detail of the surface of a cluster of cells with an apical brush border directed toward the culture medium; and *c*, intercellular lumen with the presence of an intraluminal brush border (*b* and *c*, *bar*, 1.25 μm).

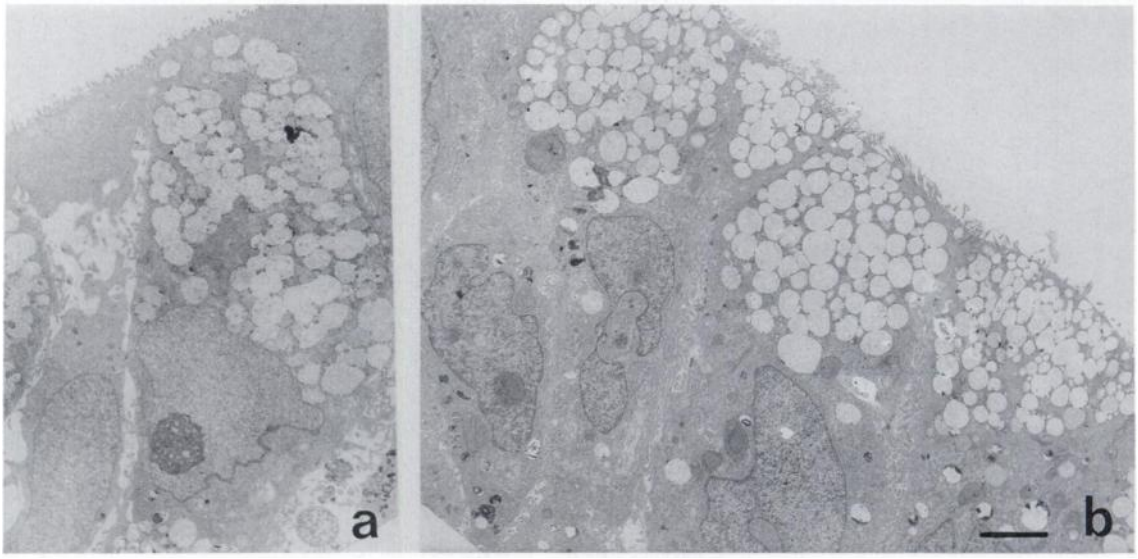

Fig. 4. Transmission electron microscopy of postconfluent cultures (Day 30) of MTX-adapted cells. Sections are perpendicular to the bottom of the flask. *a*, detail of 10^{-7} M MTX-adapted cells (passage 9) showing a columnar absorptive-like cell between two goblet cells; *b*, detail of 10^{-6} M MTX-adapted cells (passage 13) showing the exclusive presence of goblet cells. Note the presence of a discrete brush border associated with the apical cell surface of goblet cells (*bar*, 4 μm).

proportion of the same differentiated cell types as found in control cells normally passaged.

DISCUSSION

We report here the close relationship between adaptability of human colon cancer cells to methotrexate and selection of differentiated subpopulations of these cells. Differentiation is often considered as a loss of malignant potential in cancer cells. Indeed, one axis of therapeutic research strategy in colon cancers is exposition of cultured cells to "differentiating agents" such as sodium butyrate (39–42), polar solvents (41, 43–45), or retinoids (45). Recently, the use of growth factors, such as transforming growth factor β1, has been proposed (46, 47). Paradoxically, our results show that prolonged exposure of cultured cells to methotrexate gives rise to differentiated colon cancer cell populations that have the same kinetics of growth and tumorigenicity in nude mice as the original population.

The presence of cells of a differentiated phenotype, columnar absorptive as well as mucus-secreting cells, has been evidenced in the majority of colon cancers (1–11, 14) and in some cell lines (15, 18–23), but the potential implication of these cells in

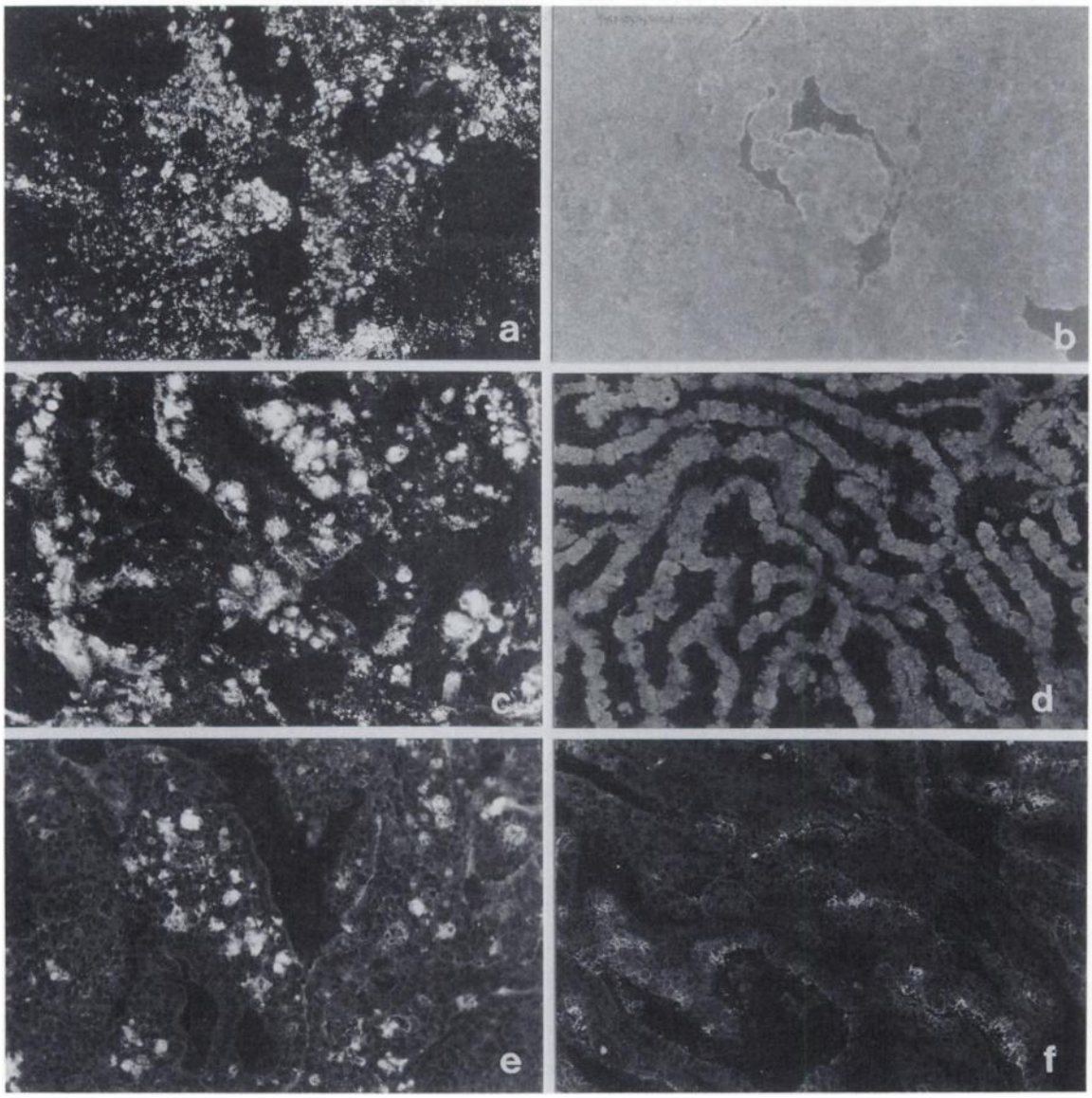

Fig. 5. Indirect immunofluorescence staining of mucins in late postconfluent cultures (30 days) of MTX-adapted cells. *a*, *c*, and *e*, 10^{-7} M MTX-adapted cells; *b*, *d*, and *f*, 10^{-6} M MTX-adapted cells; *a* and *b*, surface expression of gastric-like mucins; *c* and *d*, gastric-like mucins in cryostat sections; *e* and *f*, colonic-like mucins in cryostat sections. Results from 10^{-5} M MTX-adapted cells (not shown) were the same as in *b*, *d*, and *f*. The same results were obtained in all passages. × 85.

the acquisition of resistance to anticancer drugs has not been proposed. Our results show that stepwise acquisition of resistance to MTX is associated with cells possessing a stable differentiated phenotype. The sequence of events described for MTX is very similar to that in another metabolic stress condition, namely, glucose deprivation (26). Indeed, in both instances of metabolic stress, emergence of the differentiated subpopulation is preceded by a high rate of mortality. At the end of this slow process of adaptation, the cell culture consists exclusively of differentiated phenotypes. That these differentiated phenotypes result from selection in the heterogenous parent population of a small percentage of cells able to differentiate, rather than from an induction of differentiation, as in the malignant hematopoietic cell line HL-60 (48), is supported by a number of arguments. Selection of subpopulations adapted to MTX takes place during exponential growth (Fig. 1*A*), *i.e.*, when cells have not yet acquired the characteristics of differentiation, whether they are committed to differentiation or not (18–22, 26, 28, 29). That both types of exponentially growing cells are, however, different is supported by the observation that HT-29 cells committed to differentiation have a normal pattern of glycoprotein processing, while this pattern is impaired in cells which will not differentiate (49). Although this observation is restricted to a particular metabolic pathway, it suggests that other properties may be associated with committed, but not uncommitted, cells, which would explain their growth adaptability to MTX. Moreover differentiation of MTX-adapted cells is a stable phenomenon, persisting when the cells are reverted to drug-free medium. Finally MTX has no effect on differentiation when cells are treated after confluency (Fig. 8). Although the above arguments indirectly imply that MTX selects preexisting subpopulations, further investigation is needed for final evidence. More precisely, further experiments should investigate whether differentiated HT-29 cell clones (21, 22) and subpopulations, like those selected by glucose deprivation (18, 26), will require less adaptation to MTX than does the original population, whereas undifferentiated cell clones would not survive. Along the same lines, further studies should investigate whether MTX-adapted cells are more resistant than the original population to other chemotherapeutic agents. Researchers should

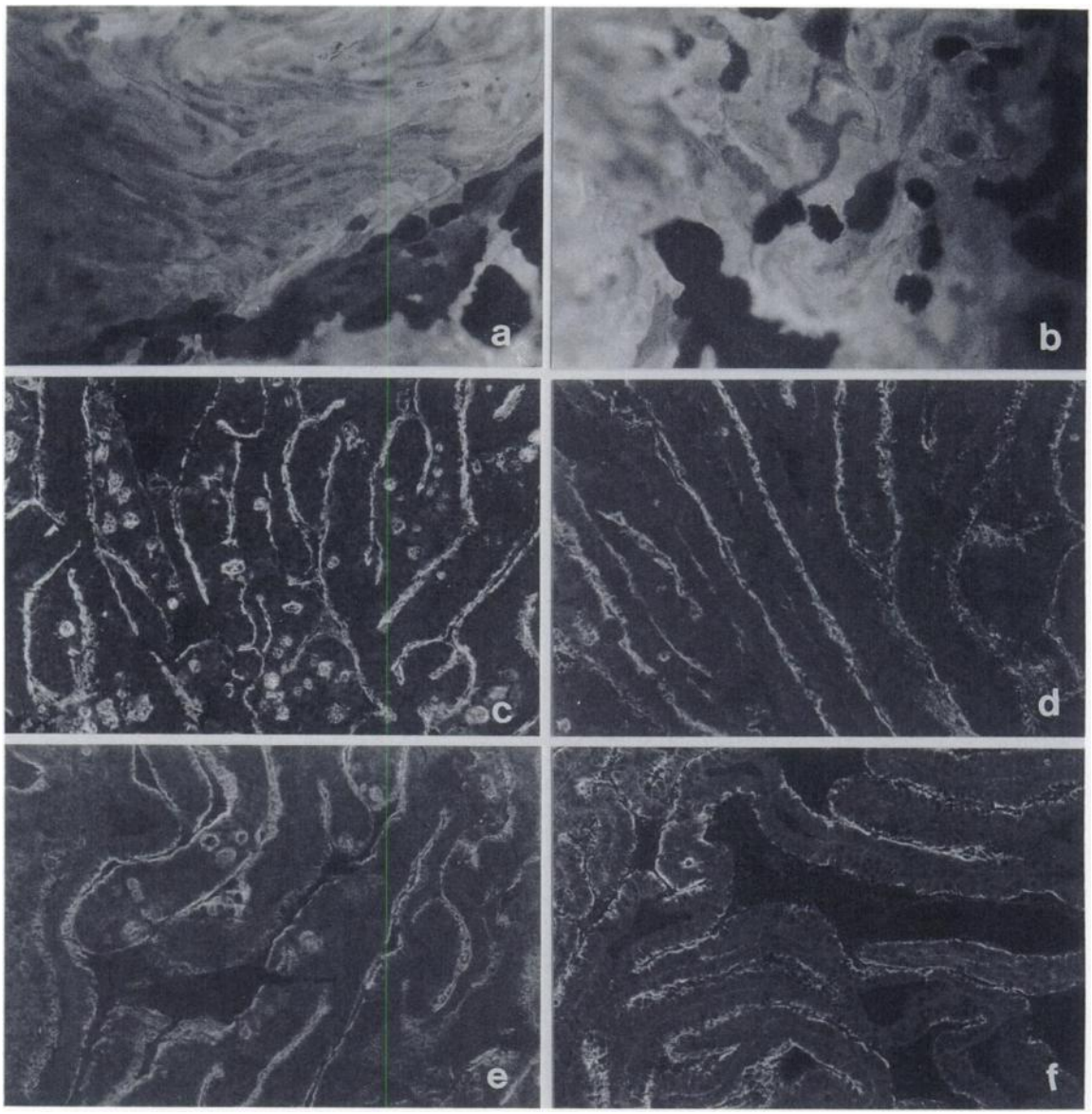

Fig. 6. Indirect immunofluorescence staining of brush border-associated proteins in postconfluent cultures of MTX-adapted cells as in Fig. 5. *a*, *c*, and *e*, 10^{-7} M MTX-adapted cells; *b*, *d*, and *f*, 10^{-6} M MTX-adapted cells; *a* and *b*, surface expression of DPP-IV on Day 21 (× 210); after 30 days the totality of the cell layer is stained (not shown); *c* and *d*, apical expression of DPP-IV in cryostat sections of the cell layer (Day 30); *e* and *f*, apical expression of villin in cryostat sections of the cell layer (Day 30). Expression of CEA (not shown) was as DPP-IV. Neither sucrase-isomaltase nor P-glycoprotein was detected. The same results were obtained in all passages (*c* to *f*, × 85).

Table 2 *Activities of brush border-associated hydrolases in HT-29 cells adapted to MTX*

Hydrolases	Control untreated cells (P145–P200)[a]	Cells adapted to 10^{-7} M MTX		Cells adapted to 10^{-6} M MTX		Cells adapted to 10^{-5} M MTX	
		MTX+[b] (P7–P22)	MTX−[c] (P1–P35)	MTX+ (P4–P23)	MTX− (P1–P17)	MTX+ (P3–P11)	MTX−
Sucrase	ND[d]	ND	ND	ND	ND	ND	Not done
DPP-IV	20.3[e]	142	100	118	120	118	Not done
	87[f]	1328		914		934	
APN	22.4[e]	23.5	21	21.7	18	25.2	Not done
AP	0.97[e]	1	0.7	1.25	1.17	1.2	Not done

[a] Enzyme activities were measured after 30 days in culture every other passage within the range of the indicated passages (P). For MTX− cells, P1 refers to the first passage in the absence of the drug.
[b] Cells adapted to MTX grown in the presence of the drug.
[c] The same cells reverted to drug-free culture medium after 8 and 6 passages for cells adapted to 10^{-7} M MTX and 10^{-6} M MTX, respectively.
[d] ND, not detectable; APN, aminopeptidase N; AP, alkaline phosphatase.
[e] Mean values of enzyme activities (see "Materials and Methods") in the cell homogenates. The SD (not shown) is less than 10%.
[f] Mean values of enzyme activities in a membrane-enriched fraction (see "Materials and Methods"). The SD (not shown) is less than 10%.

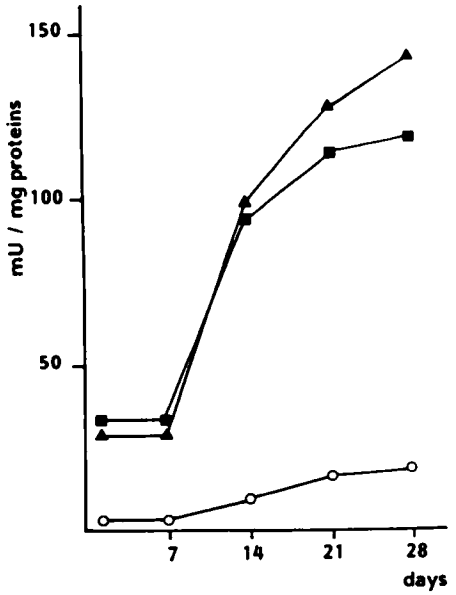

Fig. 7. Growth-related kinetics of DPP-IV activities in the cell homogenates of control HT-29 cells (O) and in cells adapted to 10^{-7} M MTX (▲) and 10^{-6} M MTX (■). *Points*, mean of 3 different cultures. The SD (not shown) is less than 5%.

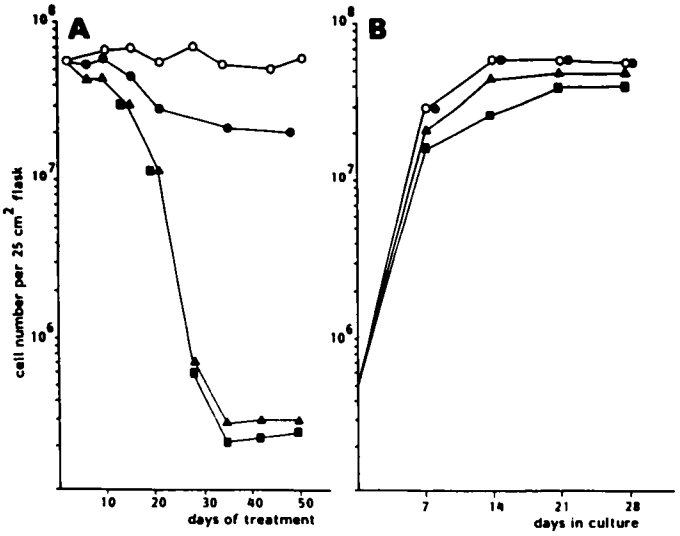

Fig. 8. Effect of MTX on stationary HT-29 cells. In *A*, postconfluent cell cultures (14 days) were maintained for 50 days in either the absence (O) or the presence of 10^{-7} M MTX (●), 10^{-6} M MTX (▲), and 10^{-5} M MTX (■). *B*, growth curves of the same cells as in *A* harvested after 50 days and cultured in drug-free medium either directly (control and 10^{-7} M MTX-treated cells) or after an intermediate 1-wk passage because of the small number of remaining cells (10^{-6} M and 10^{-5} M).

Table 3 *Rates of glucose consumption, lactic acid production, and glycogen content in control and MTX-adapted cells*

Measurements were done in postconfluent cultures (Day 21). The same results were obtained in the same cells reversed to drug-free medium.

	Control cells	Cells adapted to MTX		
		10^{-7} mol	10^{-6} mol	10^{-5} mol
Glucose consumption (nmol/h/mg of protein)	670 ± 55[a]	333 ± 25	216 ± 18	127 ± 10
Lactic acid production (nmol/h/mg of protein)	1200 ± 20	680 ± 40	400 ± 20	250 ± 15
Glycogen content (μg/mg of protein)	34 ± 2	34 ± 2	47 ± 3	51 ± 3

[a] Mean ± SD of 5 different passages.

also investigate whether drugs that are not antimetabolites but act through some other mechanism (*e.g.*, vinblastin, cysplatinum) would also result in the selection of differentiated cell populations. Despite the parallels between MTX-adapted and glucose-deprived cells, it should be noted that the type of differentiation is different. Glucose-deprived cells are a single population of absorptive cells (18, 20, 27), whereas cells adapted to low-dose MTX consist of a double population of columnar absorptive and mucus-secreting cells and, at a higher dose, of mucus-secreting cells exclusively. Interestingly the type of differentiation obtained with MTX resembles that already described for fetal colon (7, 8, 13, 14) and terminal ileum, where immunoreactivity to both mucins and hydrolases is supported by the same cells.[4] The absence of expression of sucrase-isomaltase, one of the enzymatic markers of colon cancer cell differentiation (4, 14, 25), is consistent with recent evidence that glucose, which is present in the culture medium of MTX-adapted cells, acts as an inhibitor of the biosynthesis and intracellular processing of this enzyme (27, 50–52).

Differentiation under metabolic stress conditions may be a more general phenomenon. The Caco-2 cell line, which is the only known cell line to be spontaneously totally differentiated (19), was established from a patient who had previously received anticancer chemotherapy with 5-fluorouracil.[5] Moreover, emergence of differentiated subpopulations was recently observed in HT-29 cells adapted to 5-fluorouracil and in GEO cells (20) adapted to glucose deprivation, MTX, and 5-fluorouracil.[6]

The mechanisms involved in the selection of differentiated cell types by severe metabolic stress conditions are unknown. In MTX-adapted cells, as in cells adapted to glucose deprivation (26), the rate of aerobic glycolysis is decreased. In the latter case it has been shown that an increased activity of the key enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase, is associated with the differentiation of the cells (53). We can therefore surmise that cells adapted to metabolic stress conditions, including MTX, develop metabolic advantages that allow them to escape metabolic pressure. Whether some metabolic advantage is associated with the ability of the cells to differentiate has to be further investigated. The different mechanisms involved in cell resistance to MTX have been fully documented (54) and are currently under investigation in our laboratory, along with those involved in the adaptation to other metabolic stress conditions. A crucial question that must be answered is whether a common mechanism, which would be associated with the ability of the cells to differentiate, is responsible for cellular adaptation to such different conditions as MTX, glucose deprivation, or others. The multidrug resistance gene product P-glycoprotein (55) does not appear involved. Although low levels of P-glycoprotein have been reported in HT-29 cells (56), we were unable to detect its presence in either untreated or MTX-adapted cells with both JSB-1 and MRK-16 antibodies. Furthermore, using complementary DNA probe pCHP1 (57), we did not find any amplification of the *mdr* gene by Southern blot analysis of DNA from MTX-adapted cells (not shown). A well-documented mechanism of resistance to MTX is gene amplification of DHFR (54). Whether or not gene amplification of key enzymes involved in each metabolic

[4] T. Lesuffleur, A. Barbat, and A. Zweibaum, manuscript in preparation.

[5] J. Fogh, personal communication.

[6] T. Lesuffleur, A. Barbat, E. Dussaulx, C. Sapin, A. Kornowski, and A. Zweibaum, manuscript in preparation.

stress condition is responsible for cellular adaptation to these conditions needs to be investigated.

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