Biochemical and Biophysical Research Communications **259,** 43–49 (1999)
Article ID bbrc.1999.0711, available online at http://www.idealibrary.com on IDEAL®

Multidrug-Resistant Phenotype Influences the Differentiation of a Human Colon Carcinoma Cell Line

Odile Rimet, Aline Mirrione, and Yves Barra[1]
UPRESA-CNRS 6032, UFR Pharmacie, 27 Bd J. Moulin, 13005 Marseille, France

Received March 22, 1999

The human colon carcinoma cell line HT29-D4, which constitutively expresses a very low level of the MDR1 gene product, was made multidrug resistant by transfection with a human MDR1 cDNA from the pHaMDR1/A expression vector and selection by colchicine. Resistant clones were 3- to 15-fold resistant to colchicine and were cross-resistant to doxorubicin (3- to 4-fold). MDR1 gene expression was associated with the expression of functional P-glycoprotein (gp-170); the function was reversed by verapamil and cyclosporin A. HT29-D4 cells are able to differentiate *in vitro* by replacement of glucose by galactose in the culture medium and also to release the carcinoembryonic antigen (CEA). Under these culture conditions, MDR1 mRNA and gp-170 were always expressed and the protein remained functional. Upon galactose treatment, resistant clones were less differentiated since they showed a heterogeneous monolayer organization accompanied by heterogeneous staining of cell-surface CEA and a high decrease (60–90%) of CEA release.

P-glycoprotein (gp-170), the product of the human MDR1 gene, is expressed in many normal epithelial cells particularly enterocytes and colonocytes. The protein is present on the apical membrane of intestinal cells and its ability to transport a variety of hydrophobic molecules across the plasma membrane by an ATP-dependent process has been well studied (1, 2). The high level of gp-170 expression in some tumor cells after an anticancer chemotherapy is one of the molecular bases of multidrug resistance (MDR), since the drug efflux mediated by gp-170 reduced intracellular drug concentration. In colorectal tumor cells, the relatively high expression of gp-170 might be the cause of their resistance to the majority of anticancer drugs.

[1] To whom correspondence should be addressed. Fax: (+33) 4 91 80 26 12. E-mail: yves.barra@pharmacie.univ-mrs.fr.

There is no clear relation between the level of MDR1 expression and the differentiation characteristics of the tumor. Indeed, high levels were observed in well-differentiated colorectal carcinomas (3, 4) whereas gp-170 was predominantly detected in invasive colon tumor cells (5), i.e., in less differentiated cells; but this overexpression was not associated with a worse clinical outcome (6). In cell lines, there is also controversy as to the exact association between the expression of the MDR phenotype and cell differentiation. The basal expression of MDR1 gene and/or gp-170 was generally modulated by addition of differentiating agents such as sodium butyrate, DMSO, pyruvate, retinoic acid, activin A and phorbol esters (3, 7–13). When exposing to differentiating inducers, an overexpression of MDR1/gp-170 was described in certain human colon carcinomas (3, 8) and neuroblastoma cell lines (7). However, this induction was not always associated with decreased cytotoxic drug accumulation. Another work on colon carcinoma cells reported an association between an MDR1 expression increase and the process of differentiation (14). In contrast, a downregulation of gp-170 by a differentiation factor (activin A) was described in erythroleukemia cells selected for vincristine resistance (12) and MDR myeloid leukemia cells were shown to be insensitive to phorbol ester-induced differentiation (13). Other reports concluded in a lack of influence of differentiating agents on MDR1 expression in colon cell lines (9, 10). The majority of these papers described the effect of differentiating inducers on gp-170 expression and function.

However, studying the influence of MDR1 expression on cell differentiation is less usual. Our aim was to highly increase MDR1 gene expression in human colon carcinoma cells (HT29-D4) by transfection with human MDR1 cDNA and to study the consequences of this overexpression on cell organization. In fact, the HT29-D4 clone can exist in a nonorganized state, when cultured in the presence of glucose, or in a well-polarized state in glucose-free galactose containing medium (15). Membrane expression and release of high

0006-291X/99 $30.00

levels of the carcinoembryonic antigen (CEA) occurred in polarized cells and was therefore used as an index of HT29-D4 cell differentiation (16). In this paper, we have studied the differentiation characteristics of HT29-D4 cells overexpressing gp-170, cultured in glucose or galactose containing media.

MATERIALS AND METHODS

Cell culture. The human colon carcinoma HT29-D4 cells (15) were cultured in DMEM containing 4.5 g/liter D-glucose supplemented with 10% fetal calf serum (FCS) (glu-medium). Multidrug resistant HT29-D4 cells (MDR) were grown in this glu-medium containing 6 ng/ml colchicine. To induce an organized state (16), glu-medium was substituted during 8 and 28 days by DMEM containing 0.9 g/liter D-galactose instead of glucose and 10% dialyzed FCS (gal-medium). During this gal-medium culture, cells were also treated with 20 μM verapamil (Vrp).

Isolation of HT29-D4 multidrug resistant cells by transfection with pHa-MDR1/A. HT29-D4 cells were stably transfected by pHa-MDR1/A (generously provided by Dr. Gottesman, Bethesda-USA) using lipofectin reagent according to the manufacturer's procedure (Life Technologies). Subcultures were then grown in glu-medium supplemented with 6 ng/ml colchicine. Resistant clones were isolated after 19 days. For further studies, two of them HT29-D4-MDR17 and HT29-D4-MDR31 (abbreviated as MDR-17 and MDR-31) were chosen.

Drug cytotoxicity. Cell sensitivity to colchicine and doxorubicin was measured with a MTT assay. After 48 h treatment with varying drugs concentrations, the 50% inhibitory concentration (IC_{50}), defined as the drug concentration which resulted in a 50% reduction in cell survival, was classically calculated.

MDR1 gene expression. Total RNA was extracted from cells using the RNAxel kit (Eurobio-France). One μg of total RNA was reverse transcribed using the Gibco BRL-M-MLV reverse transcriptase (RT) and random primers at 37°C for 1 h. The MDR1 and the internal control beta$_2$-microglobulin (β_2-m) cDNA were amplified with *Taq* polymerase (Appligene) and corresponding primers (17). A negative control consisting in omission of the cDNA was performed. PCR was carried out in a Perkin–Elmer system 2400. A precycle started the reaction with 3 min at 93°C, 30 s at 57°C, and 45 s at 70°C. 28 cycles followed for both genes with 10 s at 91°C, 30 s at 57°C and 45 s at 70°C. The amplification reaction was finished with an extension cycle at 70°C for 10 min. The PCR products were separated on 2% agarose gel, visualized by ethidium bromide staining and images were digitalized.

Gp-170 protein expression. For immunoblotting, membrane fractions were prepared by differential centrifugations and proteins were separated in a urea 5.6% polyacrylamide gel then transferred to a nitrocellulose filter. Gp-170 was detected using fluorescein labeled C219 monoclonal antibody (Centocor). The blots were incubated with peroxidase conjugated anti-fluorescein antibody and visualized with ECL system (Amersham).

For flow cytometry, cells, washed in phosphate-buffered saline (PBS), were incubated in human serum for 30 min at 4°C (18), then rinsed again and incubated for 30 minutes at 4°C with or without monoclonal antibody UIC2 (anti-gp-170 mouse IgG2a, Immunotech-France). After washes with PBS–BSA (1%), a 30-min incubation was performed at 4°C in the dark with fluorescein isothiocyanate-labeled (FITC) goat anti-mouse immunoglobulin (Fab)$_2$ fragment (Amersham) (1/20 dilution). Washes in PBS–BSA and fixation in PBS containing 1% formaldehyde followed. Assay in the absence of primary antibody was used as control. Analysis was performed on a FACScan Becton–Dickinson cytometer. Results were expressed as relative fluorescence intensities resulting from differences between experimental and control assay mean values.

Gp-170 protein function. Cells were incubated with rhodamine 123 (Rh-123) for 20 min at 37°C (19). In a parallel experiment, cells were incubated, before Rh-123 action, 10 min in the presence of gp-170 inhibitors [100 μM Vrp or 6 μM cyclosporin A (CsA)]. The cells were then rinsed in cold PBS and immediately analyzed by flow cytometry. Cells that had not been exposed to Rh-123 were used as controls.

Electron microscopy. Confluent cells grown during 35 days in gal-medium were fixed, dehydrated and embedded in Epon (15). Semithin and ultrathin sections were cut perpendicularly to the plane of the cell layer. Ultrathin sections were stained and examined on a Jeol Jem 1220 instrument.

Immunofluorescence microscopy. After 28 days of culture in gal-medium, cells were washed with PBS, fixed in 3.7% formaldehyde, rinsed with PBS–glycin (20 mM) and incubated with PBS–BSA (0.5%). Incubation was then performed with primary monoclonal antibody diluted at 1/100 in PBS–BSA: mouse anti-CEA MAC 601 (Valbiotech) or rat anti-dipeptidyl peptidase IV (DPPIV) (a gift from Dr. Maroux, Marseille-France). The secondary antibody was fluorescein-conjugated goat anti-mouse (dilution 1/20) or anti-rat (dilution 1/100), respectively for CEA and DPPIV. Cells were observed with an Olympus fluorescence microscope.

CEA release measurement. Twenty-four hours before each CEA determination, culture medium was changed. Then, the culture medium, collected and centrifuged 10 min at 10,000*g*, was tested for CEA amount using an enzyme immunoassay kit (EIA-CEA, Cis BioInternational). Experiments were performed a minimum of three times with similar results and numbers given were average values of triplicate determinations with less than 5% deviations in a representative experiment.

RESULTS AND DISCUSSION

The purpose of this work was to evaluate a possible effect of gp-170 overexpression on HT29-D4 cell organization in culture, upon growth in gal-medium, and on CEA release considered as a biochemical differentiation parameter. We chose the human colon carcinoma cell line HT29 since it was described to develop some of the characteristics of the normal intestinal epithelium in altered nutritional conditions (glucose-free medium) without requiring exogenous differentiating agents (15, 20). We could only detect a very low level of MDR1 mRNA in the HT29-D4 clone (not shown) using RT-PCR with high cycle number (35 cycles). We therefore stably transfected HT29-D4 cells with pHa-MDR1/A and gp-170 overexpressing clones were selected in the presence of 6 ng/ml colchicine (21), which definitely killed the parent line. Two of them with the highest (MDR-31) and the lowest (MDR-17) gp-170 mRNA levels (Fig. 1A) were chosen for further studies. With C219 antibody, immunoblots from membranes of transfected cells indicated a 170-kDa band characteristic of the glycoprotein; this band was not present in parental cells (not shown). UIC2 antibody, directed towards an external epitope (22), allowed the detection of the cell surface exposed protein by FACS (Fig. 2). gp-170

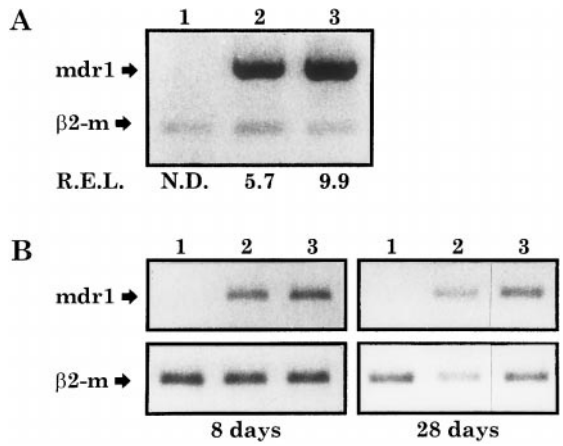

FIG. 1. RT-PCR analysis of MDR1 expression. Cells were cultured in glu-medium (A) and 8 or 28 days in gal-medium (B). Total mRNA was isolated from HT29-D4 (lane 1), MDR-17 (lane 2) and MDR-31 cells (lane 3). Relative expression level (R.E.L.): level of MDR1 mRNA normalized to β2-m.

was undetectable in HT29-D4 cells and its expression level was five fold higher in MDR-31 than in MDR-17 (Table 1). Gp-170 function, evaluated by Rh-123 assay, was correlated with mRNA and protein levels. The highest mRNA and gp-170 levels, as well as the most efficient Rh-123 efflux, were observed with MDR-31 cells (Table 1). The effects of two widely used gp-170 inhibitors (Vrp and CsA)

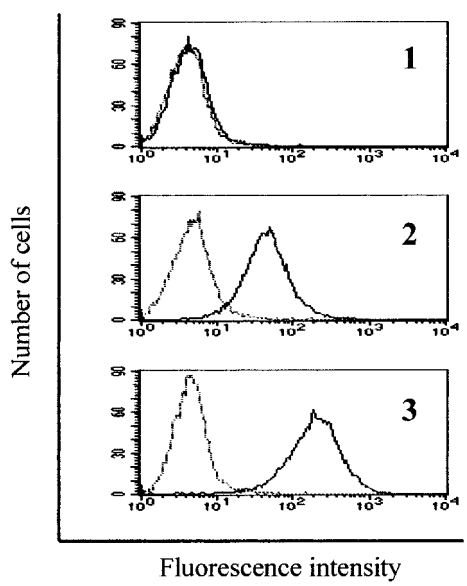

FIG. 2. FACS analysis of P-glycoprotein content in cells cultured in glu-medium. HT29-D4 (1), MDR-17 (2) and MDR-31 (3) were stained with monoclonal antibody UIC2 (solid line) and controls without antibody were performed (dashed line). The secondary antibody was a fluorescein isothiocyanate-conjugated goat anti-mouse Fab fragment.

TABLE 1

Gp170 Quantification and Functional Determination on HT29-D4 and MDR Transfectants Grown in glu-Medium and gal-Medium

Culture conditions	Assay	Relative fluorescence intensity (log) HT29-D4	MDR-17	MDR-31
Glu-medium	gp-170 (FITC)[a]	N.D.	42	211
	rhodamine-123[b]	751	452	178
Gal-medium				
(8 days)	gp-170 (FITC)[a]	N.D.	18	80
(28 days)	gp-170 (FITC)[a]	N.D.	12	43
(28 days)	rhodamine-123[b]	1173	416	258

Note. Cells grown in glu-medium were analyzed in the exponential phase of culture and cells grown in gal-medium were analyzed at confluency, as described under Materials and Methods. N.D., not detectable.

[a] FACS analysis of cells tagged with the monoclonal antibody UIC2 and detected with fluorescein isothiocyanate-conjugated goat anti-mouse Fab fragment as secondary antibody.

[b] FACS analysis of Rh-123 steady state level.

were tested on protein function. As expected, Rh-123 fluorescence levels were unchanged in parental cells whereas, in both MDR transfected cells, a 40 and a 50% increase were observed for Vrp and CsA respectively. Drug resistance was also checked (Table 2): the IC_{50} of colchicine for transfected cells was much higher than for the parental cells. Moreover, they showed cross-resistance to the anticancer drug doxorubicin. The doubling time (35 h) was not greatly changed upon MDR transfection.

The relationship between gp-170 overexpression and the degree of differentiation remains unclear. Indeed, gp-170 expression and *in vitro* cell differentiation were sometimes not related (9, 10) and sometimes positively (3, 8, 14) or negatively (12) correlated. In these reports, the authors generally analyzed the effect of several differentiating agents on gp-170 expression and function. With our approach, we could not study the influence of differentiation on MDR1 expression because of transfection. Indeed, in the plasmidic construction

TABLE 2

Drug Cytotoxicity on HT29-D4 and MDR Transfectants

IC_{50} (ng/ml)	HT29-D4	MDR-17	MDR-31
Colchicine	7	21	96
Doxorubicin	330	1180	1390

Note. Cells were cultured 2 days in glu-medium with various concentrations of drugs. Drug sensitivity is expressed as an IC_{50} value defined as the concentration required to reduce cell number by 50% compared with vehicle control of an MTT assay.

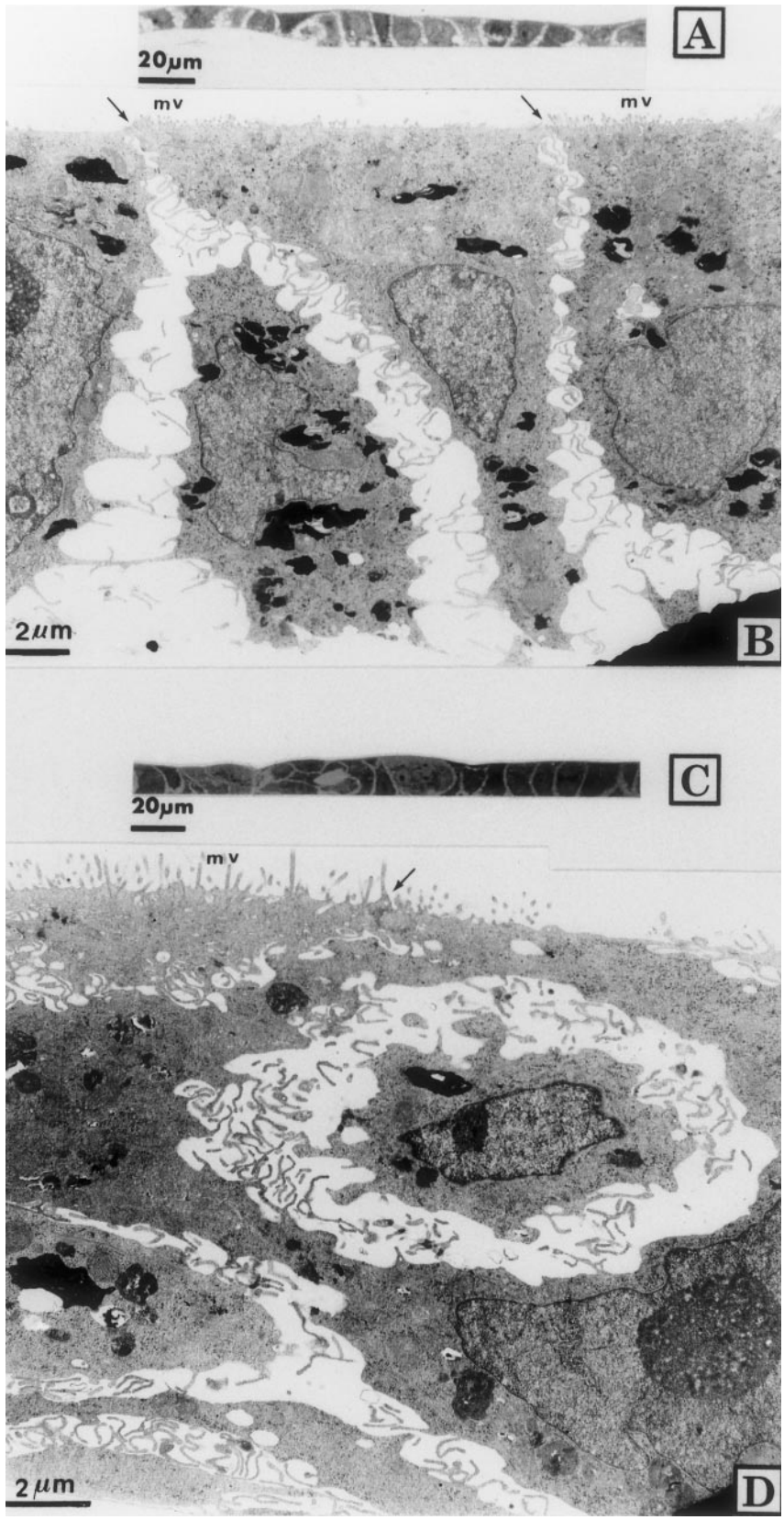

FIG. 3. Cell organization after 35 days of culture in gal-medium. For HT29-D4 cells, semithin sections microscopy (A) showed cell monolayer and electron microscopy (B) showed polarized cells with microvilli (mv) and numerous tight junctions (arrows). For MDR transfectants, semithin sections microscopy (C) showed monolayers associated with multilayers and electron microscopy (D) showed a multilayer area of cell with irregular microvilli and few tight junctions.

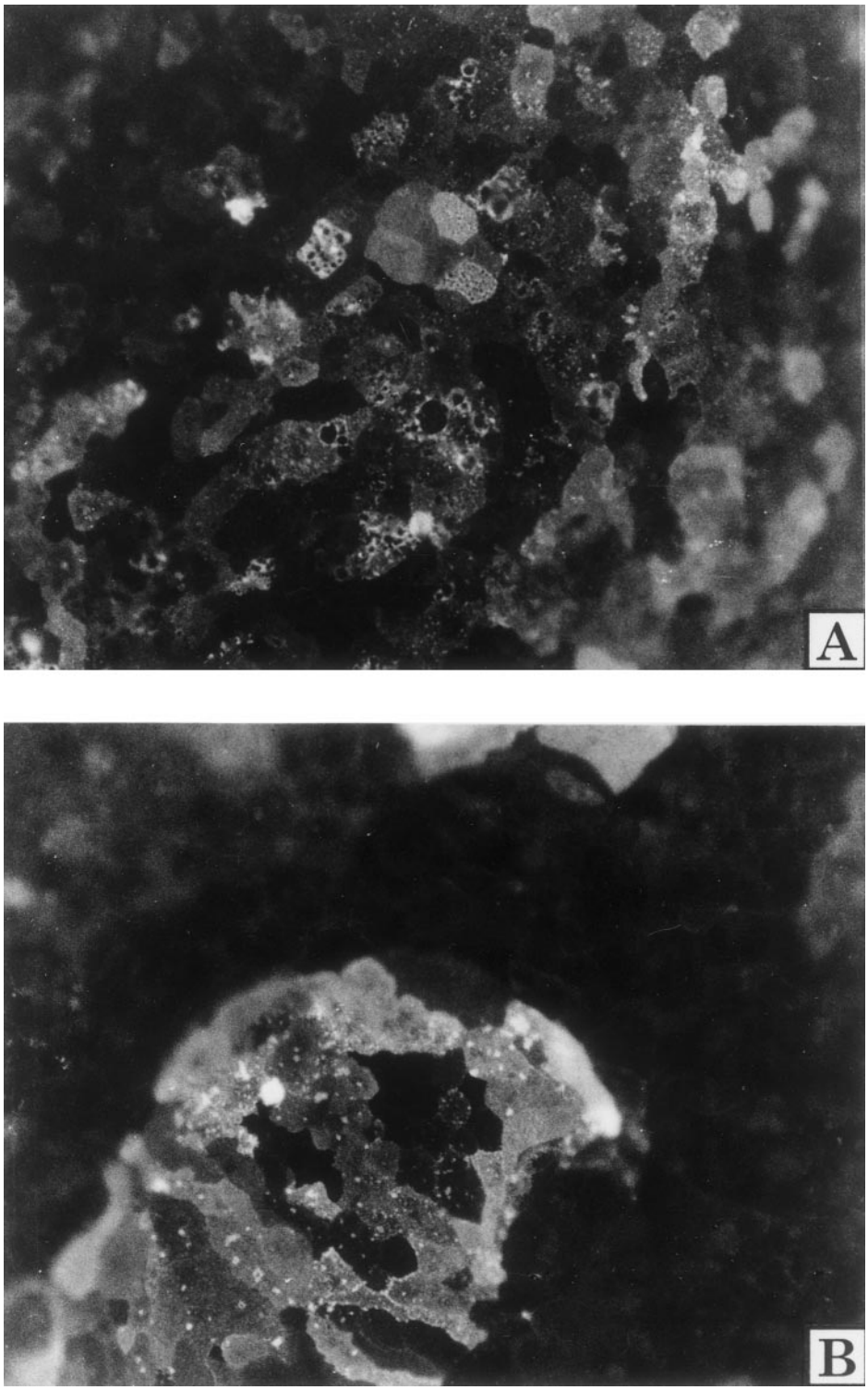

FIG. 4. Indirect immunofluorescence staining of CEA in cells cultured during 28 days in gal-medium. Cells were fixed without membrane permeabilization as described under Materials and Methods. HT29-D4 (A) and MDR transfected cells (B) were stained with monoclonal antibody anti-CEA MAC 601. Magnification, 20×.

used, MDR1 cDNA is under a viral promoter control, thus, during the differentiation process in gal-medium, the expression level cannot reflect the endogenous modulation of the MDR1 promoter. We just verified that, in transfected cells, MDR1 was always expressed when glucose was substituted by galactose. HT29-D4, MDR-17 and MDR-31 cells were thus cultured in gal-medium for 8 and 28 days. MDR1 mRNA levels were also detectable under these culture conditions (Fig. 1B) and gp-170 was always present at the cell surface (Table 1) but the levels were lower than on cells cultured in glu-medium. Moreover, the protein was functional (Table 1).

When grown in glu-medium, parental HT29-D4 as well as transfected cells formed a multilayer of morphologically unpolarized cells. As previously described, the replacement of glucose by galactose in the culture medium induced an enterocytic differentiation of HT29-D4 cells (15). At confluency, cells became polygonal, well delimited and domes appeared in the cellular monolayer (Fig. 3A). The cells appeared to be polarized with a brush border and tight junctions (Fig. 3B). On the contrary, MDR-17 and MDR-31 cells showed a heterogeneous organization when cultured in gal-medium (Fig. 3C): at late confluency, some places were organized in a monolayer of polarized cells whereas elsewhere in the dish multilayers with irregular microvilli on the upper cell layer were observed (Fig. 3D). The morphological consequence of culturing parental and resistant HT29-D4 cells in the presence of galactose was that formation of polarized monolayer was partially inhibited in the two MDR1 transfected clones. This apparent antagonism between MDR and differentiation was previously described (12, 13).

The mechanism of the organization of HT29 cells or derived clones (HT29-D4, HT29-18) into a polarized monolayer of enterocyte-like (15, 23) has still not been completely elucidated. This required the coordinated expression of genes characteristics of epithelial cells, coding for proteins involved in the epithelial organization such as integrins or cadherins, and others, more specifics of the enterocytic differentiation, e.g., DPPIV, sucrase isomaltase. CEA, a membrane bound glycoprotein expressed in several epithelial tissues, belong rather to the first group of genes but its expression has been widely used to assess the differentiation state of colic cells (8, 16). As already described for HT29-D4 cell line (16), the apical surface CEA staining was not uniform. Moreover, on individual positive cell, CEA was patchy (Fig. 4A). For both MDR transfectants, we observed areas with the same CEA fluorescence pattern as in HT29-D4 cells and areas with only slight fluorescence at the cell periphery, which is typical of undifferentiated cells (Fig. 4B). The distribution of DPPIV was more uniform on the apical poles of positive cells but, concerning differences between parental and transfected cells, results identical to those of CEA were found (not shown).

TABLE 3

CEA Release by HT29-D4 and MDR Transfectants Grown in gal-Medium

CEA ($ng/10^6$ cells/24 h)	HT29-D4		MDR-17		MDR-31	
	[a]	Vrp[b]	[a]	Vrp[b]	[a]	Vrp[b]
Day 8	51	64	5	8	21	27
Day 28	386	391	29	54	110	214

Note. The culture medium of cells cultured 8 or 28 days in gal-medium was centrifuged and tested for CEA using an immunoenzymatic assay. Culture [a] without gp-170 reverser or [b] in the presence of 20 μM verapamil.

HT29-D4 cell differentiation was also estimated by CEA release, which is oriented toward the apical side of the polarized monolayer (16). Daily CEA release in the culture medium was measured for all cells cultured in the presence of glucose or galactose. With glu-medium, the level of CEA release was low (about 2 to 5 $ng/10^6$ cells/24 h) and was not modified in MDR expressing clones.

In contrast, the high level of CEA release observed in HT29-D4 cells after 8 days or even better after 28 days in gal-medium was dramatically decreased in MDR-17 and MDR-31 clones (Table 3). An average of 90% decrease was observed in MDR-17 cells. For both clones, the effect was not significantly modified up to 40 days (not shown). MDR expression level is not correlated with the magnitude of decrease of released CEA since MDR-31 was the highest gp-170 expressing clone but showed the lowest effect on the inhibition of CEA release. However, when verapamil was added to gal-medium during the 28 days period, no difference was observed for MDR1 expression of transfected cells but a doubling of CEA release (Table 3), associated with a partial reversion of the gp-170 function, was found in the two resistant clones.

These morphological and biochemical data indicated that *in vitro* differentiation was incomplete when MDR was overexpressed. Our observations could be the consequence of gp-170 overexpression or a result of cell cloning under colchicine pressure. The first hypothesis should be retained because gp-170 inhibition with Vrp resulted in partial restoration of CEA release, i.e., partial reversion of de-differentiation. CEA, present on normal cells, can be overexpressed at the surface of cancer cells and its level, when increased in blood, is considered to be an index of the occurrence of cancer especially for colon, breast or lung (24). Indeed, circulating CEA is often used to follow the presence of breast cancer metastasis. In these conditions, if such an inverse correlation between gp-170 overexpression

and decreased CEA release exists, this phenomenon, when occurring after a chemotherapy, might mask the escape of resistant cells. In addition, MDR reversal *in vivo* using gp-170 inhibitors (25), anti-gp-170 antibodies (21) or MDR1 antisense RNA (26) may have the advantages, not only to circumvent resistance and thus to increase drug efficacy, but also to antagonize the tumor dedifferentiation process.

ACKNOWLEDGMENTS

We are grateful to M. Roccabianca, C. Prevot, and F. Giannellini for expert technical assistance. We also thank J. Mauchamp and J. Marvaldi for helpful discussions.

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