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PHASE I AND II BIOTRANSFORMATIONS IN LIVING CaCo 2 CELLS CULTIVATED UNDER SERUM-FREE CONDITIONS

SELECTIVE APICAL EXCRETION OF REACTION PRODUCTS

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Abstract—CaCo 2 cells, cultivated in a synthetic, serum-free nutritive medium on poly (ethylene terephthalate) membranes, form a confluent monolayer of differentiated cells, with the apical and basolateral poles exposed to the upper and lower compartments, respectively, of bicameral culture inserts (Halleux and Schneider, *In Vitro Cell Dev Biol*, **27A**: 293–302, 1991). This cell culture system allows the passage of intact mannitol by the paracellular route and the transcellular diffusion of testosterone which appears mainly as a biotransformed unconjugated metabolite. When ethoxyresorufin is added to either the apical or basolateral poles of living CaCo 2 cells, resorufin is formed, and more than 80% is excreted at the apical pole. Under our experimental conditions, no detectable amounts of glucurono- or sulfoconjugates are found. Methylcholanthrene and phenobarbital increase the biotransformation of ethoxyresorufin 50 and 3 times, respectively, and induce that of benzoxyresorufin, but not of pentoxyresorufin which remains absent under all conditions. These substances do not affect the proportion of resorufin recovered at the apical role. Verapamil inhibits by 25% the release of resorufin but does not affect its distribution. Chlorodinitrobenzene is conjugated with glutathione and at least two-thirds of the product is excreted at the apical pole; methylcholanthrene and phenobarbital do not increase this activity. These results demonstrate that differentiated CaCo 2 cells, under serum-free conditions, perform phase I and II reactions and that the biotransformation products are selectively excreted at the apical pole.

The cellular and molecular mechanisms whereby substances interact and/or pass across the epithelial barrier of the small intestine still remain a central problem in both cell biology and pharmacology. This is particularly important with the actual development of new strategies to improve the delivery of existing molecules, new drugs (synthetic molecules, peptides, . . .) or new vehicles (nanoparticles, liposomes, proteins, . . .). As reviewed recently [1, 2], there is a trend to develop cell culture systems as *in vitro* models of biological barriers to investigate transepithelial passage and metabolism. Appropriately validated, such systems should dramatically improve our understanding of these processes; in addition, they allow the use of cells of human origin instead of deriving from other mammals, whose gastrointestinal tract varies considerably [3].

We have set up a cell culture system as an *in vitro* model of the intestinal barrier [4]. It is based on the cultivation of CaCo 2 cells in a synthetic, serum-free nutritive medium, on extracellular maxtrix-coated poly (ethylene terephthalate) membranes. Although deriving from a human colon adenocarcinoma [5], these cells spontaneously form monolayers with a high degree of enterocytic differentiation giving a more relevant *in vitro* model than many strains or lines derived from intestinal mucosal cells. They grow and, after confluency, expose their apical pole with microvilli to the upper compartment of the bicameral culture insert whereas the basal pole is attached to the microporous membrane in contact with the lower compartment [4, 6]. Differentiated cells synthesize transferrin, transferrin receptor, fibronectin, apolipoprotein B, . . . and vectorially secrete most of them [4]. They also absorb iron at their apical pole and release most of it at the basolateral pole under different forms (Halleux and Schneider, *J Cell Physiol*, in press). In comparable conditions, CaCo 2 cells keep many properties of enterocytes, such as e.g. lipoprotein secretion [7], sorting of membrane proteins [8] and nutrient/drug transport [9, 10]. They also express glutathione *S*-transferases [11, 12], cytochrome P450 [12] and realize glucurono- and sulfoconjugation [12–14].

In this paper, we report that living CaCo 2 cells, cultivated under serum-free conditions, perform, like human enterocytes, cytochrome P450-dependent oxidations (phase I reactions involved in drug metabolization or biotransformations) and conjugate xenobiotics to glutathione (phase II reactions). Furthermore, when living CaCo 2 cells are incubated with substrates of phase I and II reactions, they biotransform them and, more important, vectorially excrete the reaction products at the apical pole.

MATERIALS AND METHODS

Cell cultivation. CaCo 2 cells (American type

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culture collection, Rockville, MD, U.S.A.) have been previously adapted in synthetic, serum-free medium and are routinely maintained by repetitive subcultivation in flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.), as described previously [4, 6].

The synthetic medium is the basal defined medium (BDM*) which is prepared on a customary basis by Gibco (Life Technologies Ltd, Paisley, U.K.). It consists of a 5:5:1 (by vol.) mixture of Iscove's Dulbecco's modified Eagle's, Ham's F12 and NCTC 135 media [15, 16]. It contains 25 mM glucose, 6 mM glutamine, 0.01% (w/v) Pluronic F68, 50 μM ethanolamine, 25 mM HEPES, 3 g/L $NaHCO_3$, penicillin (30 μg/mL) and streptomycin (70 μg/mL). It is further supplemented with 1 μg/mL insulin (Gibco), 1 ng/mL epidermal growth factor (Boerhinger; Mannheim, F.R.G.), trace elements (Mn, Mo, Ni, Si, Sn and V, as a mixture from Gibco), 10 μg/mL albumin complexed to linoleic acid (as a mixture from the Sigma Chemical Co., St Louis, MO, U.S.A.), 2 nM T3 (Sigma) and 100 nM hydrocortisone (Sigma).

For the experiments, CaCo 2 cells were cultivated in cell culture inserts (Falcon 3090, Becton Dickinson Labware) adapted for six-well microplates. They contain a poly (ethylene terephthalate) microporous membrane (Cyclopore, Louvain-La-Neuve, Belgium). This membrane (5 cm^2 of growth area; 0.45 μm of pore diameter) is fully transparent and sustains the adhesion, proliferation and differentiation of CaCo 2 cells [4, 6]. Membranes were precoated with bovine skin type I collagen (Boerhinger) at 33 μg/mL in phosphate-buffered saline for 2 hr at 37°; cells are then plated at 160,000 cells/cm^2 in synthetic BDM with 2.1 mL in the upper compartment of the bicameral chamber and 2.8 in the lower one. Culture medium is changed three times per week.

For cell proliferation determination, cells were plated and cultivated as above. After different lengths of time at 37°, the culture medium was removed; cells were washed with phosphate-buffered saline and scraped in 100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.4. After sonication (15 sec, 40 W), the activity of *N*-acetyl-β-glucosaminidase was determined according to Ref. 17; cell number was then interpolated by comparison with standard curves established on cells detached with trypsin and counted by the Trypan blue exclusion test.

Transepithelial passage. Confluence of the monolayer was assayed by incubating cells with 2.1 mL of BDM in the upper compartment of the insert and 2.8 mL of phenol red-free basal Eagle's medium (Gibco) in the lower one, as in Ref. 4. After 2.5 hr at 37°, E_{546} was determined in both compartments. Results were expressed as the per cent of the dye recovered in the lower compartment; only inserts for which this value was lower than 6.5% were used. In some cases, the transepithelial electrical resistance was also assayed (Endohm 24; World Precision Instruments, Sarasota, FL, U.S.A.). All experiments were carried out with cells cultivated for 18–21 days in culture inserts.

Transepithelial passage was assayed by adding 7 μM [^{14}C]mannitol, 15 nM [^{3}H]testosterone (both from Amersham International, U.K.) or 5 μM resorufin (Aldrich, Steinheim, F.R.G.) in either the upper or lower compartment of the bicameral culture insert containing 2.1 or 2.8 mL of synthetic, serum-free BDM, respectively. After different lengths of time, ranging from 15 to 180 min, 100 and 75 μL of culture medium (to maintain the same volume ratio in both chambers) were removed from the lower and upper compartments, respectively. For the experiments at 4°, cells were preincubated for 30 min at 4° before addition of the labelled probes; for the experiments in the presence of 2.5 mM EGTA, the chelator and the probes were added simultaneously. At the end of the experiments, each individual culture insert was inspected under phase contrast microscope. A possible cytotoxic effect was ruled out by assaying the activity of lactate dehydrogenase in the culture medium of both the upper and lower compartments, according to [18]; the proportion of the activity found in the medium was systematically lower than 4% of that present in cell lysates prepared from culture inserts in 0.5% Triton X-100. Control experiments were performed with collagen-coated membranes, but in the absence of cells.

The amounts of radioactive ([^{14}C]mannitol and [^{3}H]testosterone) or fluorescent (resorufin) material were assayed in the culture medium from both compartments. Results were then calculated as clearance, i.e. after conversion of the amount of labelled material recovered in the opposite compartment to the corresponding volume (μL) of culture medium that passed across the cell monolayer. The clearances were plotted versus the duration of the experiment and a linear regression was calculated. The results were finally expressed as μL/hr and cell culture insert. The protein content (see below) was found to be largely constant from experiment to experiment (1.240 ± 0.080 mg cell protein/insert).

Biotransformation studies. The ^{14}C-labelled material that passes across the cell monolayers during incubation of the cells with [^{14}C]mannitol has been characterized by paper chromatography, with *n*-butanol/ethanol/water (52/33/15, by vol.) as solvent. The ^{3}H-labelled material ([^{3}H]testosterone) has been analysed by TLC with toluene/ethylacetate (50/30, v/v) or cyclohexane/ethylacetate (60/40, v/v) as solvent. The culture media were chromatographed as such or after extraction with the solvents used for the separation, before or after deconjugation with β glucuronidase and arylsulphatase (see below). Chromatograms were cut into fractions and analysed by liquid scintillation. Profiles were compared to standards of unlabelled testosterone and androstenedione.

Biotransformation of resorufin ethers has been adapted from Ref. 19: after washing, CaCo 2 cells were incubated for 1 hr at 37°, as above, in phenol red-free basal Eagle's medium containing 5 μM of either ethoxy- (EROD), benzoxy- (BROD) or pentoxyresorufin (PROD) (Boerhinger) in one of the two compartments of the bicameral insert. After the incubation, the media were collected and centrifuged

* Abbreviations: BDM, basal defined medium; BROD, benzoxyresorufin; EROD, ethoxyresorufin; PROD, pentoxyresorufin.

for 15 min at 3000 rpm; fluorescence was measured on supernatants with excitation and emission wavelengths of 530 and 585 nm, respectively; results were compared to a standard of resorufin. Deconjugation was performed as in Ref. 20, by incubating for 24 hr at 37°, 500 μL of the culture medium with 500 μL of 0.2 M acetate buffer pH 5, containing a mixture of β glucuronidase and arylsulphatase (Boerhinger), in the absence of cells; pH was then brought back to a value ≥pH 9, before assay of fluorescence as above.

Conjugation of 1-chloro-2,6-dinitrobenzene to glutathione has been adapted from Ref. 21: after washing, cells were incubated for 1 hr at 37° with phenol red-free basal Eagle's medium, as above; the medium of one compartment was supplemented with 50 μM chlorodinitrobenzene. After the incubation, absorbance at 340 nm was determined.

For some experiments, 5 μM 3-methylcholanthrene (Sigma), diluted in BDM from a 0.5 mM solution in dimethyl sulphoxide, or 2 mM phenobarbital (local pharmacy), diluted in BDM from a 0.5 M solution in ethanol, were added. These two specific cytochrome P450 isozyme inducers were added to the nutritive medium, before biotransformation assay, as above. The absence of cytotoxicity was ruled out by assaying the lactate dehydrogenase activity in the culture medium, as above.

Other assays. Assay of the alkaline phosphatase activity has been adapted from Ref. 22, but directly performed on the intact living cell monolayer using *p*-nitrophenylphosphate as substrate.

The amounts of radioactive material in the fractions were determined after dispersion in 5 mL of Aqualuma cocktail (Lumac, Basel, Switzerland) in a Tri-Carb 460 CD liquid scintillation system (Packard Instruments, San Diego, CA, U.S.A.). Protein was assayed according to Ref. 23, with serum albumin as standard.

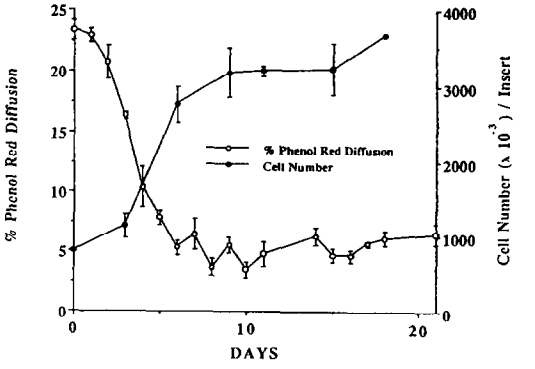

Fig. 1. Cell growth and confluence. CaCo 2 cells were plated and cultivated on collagen-coated poly (ethylene terephthalate) microporous membranes in synthetic medium. Confluence of the cell monolayer was assayed by the phenol red diffusion test. Cell number was estimated from determination of the activity of *N*-acetyl-β-glucosaminidase, by comparison to a standard curve. Protocols are described in Materials and Methods. Mean results of six independent experiments ± SEM are given.

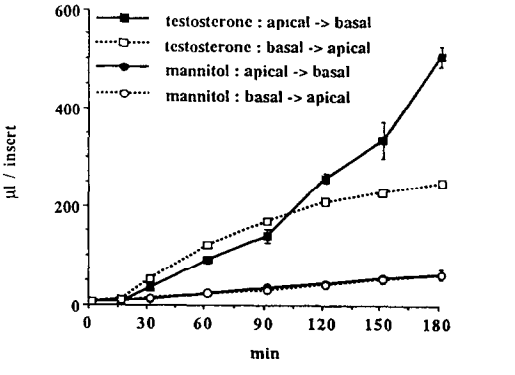

Fig. 2. Transepithelial passage of [^{14}C]mannitol and [^{3}H]-testosterone across CaCo 2 monolayers. [^{14}C]Mannitol and [^{3}H]testosterone were added to either the upper or the lower compartment of the culture insert at 7 μM and 15 nM, respectively. After different lengths of time of incubation at 37°, the amounts of radioactive material were assayed in the opposite compartment, as described in Materials and Methods. Mean results of eight independent experiments ± SEM are given.

RESULTS

Cell growth and confluence

Upon plating on collagen-precoated poly (ethylene terephthalate) membranes, in a serum-free nutritive medium, the number of CaCo 2 cells increases after a lag period and reaches a plateau after 9 days (Fig. 1). These results are confirmed by assay of the amount of protein or DNA associated with the substrate (not illustrated). Phase contrast microscopy further indicates that cell confluence is attained after about 1 week. The rate of passage of phenol red across the cell layer dramatically falls from the day of inoculation to days 6–7, where it reaches a low and stable value (Fig. 1). Concurrently, the transepithelial electrical resistance attains a stable value (350 ± 20 ohm × cm^2) which remains constant for up to at least 3 weeks.

Transepithelial passage

Figure 2 illustrates the transepithelial passage of ^{14}C and ^{3}H labels, upon incubation of confluent CaCo 2 cells with [^{14}C]mannitol and [^{3}H]testosterone, added to either the upper compartment (mimicking the apical pole of the intestinal cell) or the lower compartment (basolateral pole) of the bicameral cell culture insert. The passage of ^{14}C-labelled material proceeds at a rate proportional to the duration of the incubation for up to, at least, 3 hr. The clearance rate is almost equal from the upper to the lower compartment and vice versa, reaching a value of 18 μL/insert × hr (Table 1). Analysis by paper chromatography reveals that ^{14}C label migrates as a single peak, with the same R_f as mannitol (not illustrated).

After a lag period, ^{3}H label also appears in the opposite compartment (Fig. 2): during an initial phase of *ca.* 90 min, clearance is comparable in both

Table 1. Transepithelial passage of [^{14}C]mannitol and [^{3}H]testosterone in CaCo 2 cells

Experimental conditions	Transepithelial passage* of [^{14}C]mannitol: Apical to basolateral	[^{14}C]mannitol: Basolateral to apical	[^{3}H]testosterone: Apical to basolateral	[^{3}H]testosterone: Basolateral to apical
Control	18.5	17.7	88.0†	111.7†
			229.0	52.5
Absence of cells‡	211.1	195.8	203.0	205.0
4°	14.8	12.2	58.9	46.1
+2.5 mM EGTA	147.2	106.1	212.9	208.9
+50 μg/mL cycloheximide	18.1	ND§	130.5	152.1

* All the results of transepithelial passage are expressed as μL/culture insert × hr, after calculations from experimental data by linear regressions (regression coefficients were ≥0.96); results represent means of eight independent cell culture inserts which all involved determination of the passage after seven time intervals ranging from 15 to 180 min.

† The first figure corresponds to the clearance calculated from the figures obtained from 0 to 90 min, whereas the second one represents the rate calculated from 90 to 180 min.

‡ Experiments were carried out as above, but with collagen-precoated microporous membranes, in the absence of cells.

§ ND, not done.

directions, although weaker from the apical to the basolateral pole (88 μL/insert × hr; Table 1) than in the opposite direction (112 μL/insert × hr). In a second phase, the clearance rate slows down from the basolateral to the apical pole (53 μL/insert × hr), whereas it considerably increases in the opposite direction (229 μL/insert × hr). The clearance of testosterone appears about 12 times higher than that of mannitol.

Table 1 indicates that the presence of CaCo 2 cells dramatically restricts the passage of [^{14}C]mannitol, dividing the diffusion rate by a factor of more than 11, whereas this effect is much more reduced in the case of [^{3}H]testosterone. At 4°, a temperature at which energy-dependent processes are considerably slowed down, the transepithelial passage of [^{14}C]-mannitol is decreased by only 20–30%, whereas clearance rates of [^{3}H]testosterone are decreased to *ca.* 50 μL/insert × hr. EGTA, a calcium chelator known to open intercellular tight junctions and which, under our experimental conditions, decreases the transepithelial electrical resistance to values ≤50 ohm × cm^2, multiplies the clearance rate of [^{14}C]mannitol by respectively 8 (apical→basolateral) and 6 (basolateral→apical) whereas it has no effect on [^{3}H]testosterone. Finally, cycloheximide, a protein synthesis inhibitor, has no significant effect on the passage of [^{14}C]mannitol from the upper to lower compartment but decreases the passage of [^{3}H]testosterone.

When 5 μM resorufin is added to the upper or lower compartment of the bicameral insert, it also passes across CaCo 2 cell monolayers. From the apical to basolateral pole, a clearance rate of 223 μL/insert × hr is observed, which is almost identical to that of testosterone (second phase), whereas in the opposite direction, a value of 291 μL/insert × hr is measured, which is significantly higher than that of this hydrophobic molecule.

Biotransformation

Thin layer chromatographs (not shown) indicate that *ca.* 80% of the ^{3}H label that passed across CaCo 2 cell monolayers incubated in the presence of [^{3}H]testosterone migrates with a R_f of 0.48 (0.32 for native testosterone); *ca.* 20% migrates as native testosterone whereas no significant amount of ^{3}H label migrates as androstenedione. Neither the proportion of ^{3}H label extractable nor the migration profile was affected upon reincubation of the culture media with a mixture of β glucuronidase and arylsulfatase, suggesting the absence of conjugation of testosterone during its transport and after its biotransformation. As a control, it was checked that no biotransformation takes place in the absence of cells or with conditioned medium.

Figure 3 shows that, upon incubation with EROD, living CaCo 2 cells release resorufin into the extracellular medium. During the proliferation phase (*ca.* 7 days, Fig. 1), the amount of resorufin/mg cell protein increases by a factor of 6.4 and then remains stable after confluency; after full differentiation of the cells (*ca.* 14 days [4]; activity of alkaline phosphatase, Fig. 3), the amount of resorufin that is released by *ca.* 35% of the maximal level. *In vitro* reincubation of the culture medium in the presence of arylsulphatase and glucuronidase, to deconjugate resorufin esters, does not increase significantly the amount of resorufin. [It should be noted that when living rat hepatocytes, cultivated for 4 days under almost the same conditions, are incubated with EROD, *in vitro* deconjugation with the same protocol multiplies the amount of resorufin by a factor of 13 (Jaumotte-Thelen and Schneider, unpublished results).]

CaCo 2 cells, under conditions of confluency and differentiation, were incubated with EROD and the amount of resorufin was determined in the two

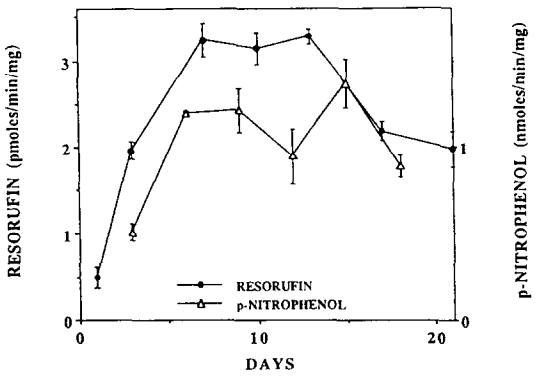

Fig. 3. Biotransformation of ethoxyresorufin by living CaCo 2 cells. Cells were inoculated and cultivated in culture inserts as described in Materials and Methods. After different lengths of time of cultivation, culture medium was replaced by phenol red-free medium and 5 μM ethoxyresorufin was added to the upper compartment. After 1 hr at 37°, medium from the upper and lower compartments was removed, pooled and analysed for fluorescence. Alkaline phosphatase was assayed by measuring *p*-nitrophenol production in the medium. Results are correlated with the cell protein content of each individual insert. Mean results of three independent experiments ± SEM are given.

compartments of the culture inserts. Table 2 indicates that, upon exposure to EROD added to either the apical or the basolateral poles, 80–100% of the amount of resorufin formed by the phase I biotransformation is recovered in the upper compartment, suggesting a preferential release at the apical pole of the epithelial cells. In addition, the amount of resorufin which is formed is increased by 35% when the cells take up EROD from the basolateral pole.

When CaCo 2 cells, preincubated for 24 hr with 5 μM methylcholanthrene (a non-cytotoxic dose under our serum-free culture conditions), were exposed for 1 hr to EROD added to the apical pole, the amount of excreted resorufin is multiplied by 45 (Table 3); 2 mM phenobarbital also increases excretion of resorufin but to a lesser extent (×2.8). The proportions of resorufin recovered in the two compartments of the bicameral culture chamber are unaffected by the inducers and amounts ≥80% are recovered at the apical pole.

In the absence of inducers, BROD and PROD are not transformed into detectable levels of resorufin by CaCo 2 cells; after induction by methylcholanthrene or phenobarbital, biotransformation of BROD occurs, but resorufin is only detected at the apical pole (Table 3); even in the presence of the inducers, PROD is not transformed to detectable levels of resorufin. In all cases, *in vitro* reincubation of the culture medium with arylsulphatase and glucuronidase does not increase significantly the amount of resorufin detected (not illustrated).

P-glycoprotein is present in the apical plasma membrane domain of rat small intestinal mucosal cells [24] and in human intestinal adenocarcinoma cell lines (CaCo 2 cells [12]; HCT-8 and T 84 [25]). It is involved in the detoxification mechanisms, along with the cytochrome P450 enzymatic system, pumping toxins out of the cells into the lumen of the gastrointestinal tract. Table 4 illustrates that verapamil, a P-glycoprotein inhibitor, decreases by *ca.* 25% the release of resorufin from cells incubated with EROD, upon induction with methylcholanthrene; nevertheless the proportion of resorufin in the two compartments is unaffected by verapamil.

Table 5 shows that CaCo 2 cells conjugate chlorodinitrobenzene with glutathione; upon addition of the substrate to the upper or lower compartment of the insert, two thirds of the biotransformation product is released at the apical pole. The activity is not increased by the inducers.

DISCUSSION

This cell culture system, developed as an *in vitro* model of the intestinal barrier [4, 6], is appropriate for transport and metabolism studies. CaCo 2 cells,

Table 2. Polarized excretion of resorufin after biotransformation of EROD by living CaCo 2 cells

	Resorufin* recovered in			
	apical compartment		basolateral compartment	
Compartment of addition	pmol/min/ mg cell protein	% of total	pmol/min/ mg cell protein	% of total
Apical	2.17 ± 0.30	80.07	0.54 ± 0.08	19.92
Basolateral	3.65 ± 0.54	100	ND	0

Cells cultivated from 16 to 19 days, as described in Materials and Methods, were incubated for 1 hr at 37° with 5 μM EROD added in either the upper (apical) or lower (basolateral) compartment of the bicameral culture insert.

* Amount of resorufin released; mean results of 10 (substrate in apical compartment) or six (basolateral) independent experiments ± SEM are given.

ND, not detected.

Table 3. Effect of methylcholanthrene and phenobarbital on the biotransformation of resorufin ethers and the polarized excretion of resorufin by living CaCo 2 cells

	Resorufin* recovered in			
	apical compartment		basolateral compartment	
Substrate	pmol/min/ mg cell protein	% of total	pmol/min/ mg cell protein	% of total
EROD				
Untreated controls	1.51 ± 0.43	79.47	0.39 ± 0.12	20.53
+ Methylcholanthrene	74.36 ± 0.30	87.19	10.93 ± 0.08	12.81
+ Phenobarbital	4.41 ± 0.45	81.67	0.99 ± 0.24	18.33
BROD				
Untreated controls	ND	—	ND	—
+ Methylcholanthrene	0.32 ± 0.07	100	ND	0
+ Phenobarbital	0.10 ± 0.02	100	ND	0

Cells were cultivated for 19 days, as described in Materials and Methods; they were then preincubated or not for 24 hr with 5 μM methylcholanthrene or 2 mM phenobarbital, before addition of the substrate to the upper (apical) compartment.

* Mean results of six independent experiments ± SEM are given.

ND, not detected.

Table 4. Effect of verapamil on resorufin excretion after biotransformation by living CaCo 2 cells

	Resorufin* recovered in			
	apical compartment		basolateral compartment	
Addition	pmol/min/ mg cell protein	% of total	pmol/min/ mg cell protein	% of total
− Verapamil	75.13 ± 12.34	85.58	12.66 ± 1.92	14.42
+ Verapamil†	55.85 ± 12.49	85.28	9.64 ± 0.63	14.72
(% of control)	(74.34)		(76.15)	

Cells were cultivated from 18 days, as described in Materials and Methods; they were then preincubated for 24 hr with 5 μM methylcholanthrene before addition of EROD for 1 hr.

* Mean results of six independent experiments ± SEM are given.

† 0.2 mM verapamil was present in upper and lower compartments.

Table 5. Polarization of glutathione conjugate excretion after biotransformation by living CaCo 2 cells

	Conjugate* recovered in			
	apical compartment		basolateral compartment	
Compartment of addition	pmol/min/ mg cell protein	% of total	pmol/min/ mg cell protein	% of total
Apical	0.34 ± 0.02	66.67	0.17 ± 0.03	33.33
Basolateral	0.27 ± 0.02	65.85	0.14 ± 0.01	34.15

Cells were cultivated from 16 to 20 days, as described in Materials and Methods before addition of 1-chloro-2,6-dinitrobenzene for 1 hr at 37°.

* Mean results of six independent experiments ± SEM are given.

cultivated in a synthetic, serum-free and almost protein-free medium, adhere on extracellular matrix-coated poly (ethylene terephthalate) membranes. After 1 week, cells reach confluency and form a continuous monolayer restricting the passage of phenol red (Fig. 1) and characterized by a high transepithelial electrical resistance, abolished in the presence of EGTA. Thereafter, CaCo 2 cells differentiate, expressing brush border enzymes (Fig. 3; unpublished results) and exposing an apical pole to the upper compartment of the culture insert and a basal pole attached, via an extracellular matrix, to the microporous membrane [4]; they synthesize and vectorially secrete transferrin and apolipoprotein B [4], as well as transferrin receptor (Sergent-Engelen and Schneider, unpublished observation). Recent work from other investigators confirms that cultivated in a comparable synthetic medium, CaCo 2 cells still express specific brush border enzymes [26].

Mannitol crosses the monolayer, without apparent biotransformation, at an equal rate from the apical to the basolateral pole and *vice versa*. Clearance is unaffected by cycloheximide and only slightly reduced at 4°; in contrast, it is multiplied by a factor of 7 in the presence of EGTA, which is almost equal to that reported for a hydrophilic substance, atenolol, in the CaCo 2 model [27]. All these results are in agreement with transepithelial passage by a paracellular route, as already proposed [28–30].

Testosterone passes across the cell culture system at very high rates, which may reach those observed through collagen-precoated membranes in the absence of cells. This passage is largely inhibited at 4°, but unaffected by cycloheximide; EGTA increases the passage by *ca.* 18%, which is comparable to that reported for another lipophilic substance, propanolol [27]. As suggested previously [31], we propose a transcellular route, involving the diffusion of this hormone within cellular (apical and basolateral) membranes. In addition, the hormone that crossed the monolayer is almost entirely metabolized and this biotransformation requires the presence of cells. Recently, it has been reported that differentiated CaCo 2 cells metabolize testosterone to androstenedione [32]; under our culture conditions, the ^{3}H label that crossed the cell monolayers does not behave as this metabolite. Our data also indicate that [^{3}H]testosterone and the metabolite released are not glucorono- or sulfoconjugated.

Resorufin crosses CaCo 2 monolayers at rates comparable, although higher from the basal to the apical pole, to those of testosterone, suggesting also transcellular diffusion.

Our results demonstrate that living CaCo 2 cells perform phase I biotransformations as evidenced by O-deethylation of ethoxyresorufin; this activity increases during cell proliferation, remains stable during cell differentiation, but decreases slightly thereafter (Fig. 3). Cytochrome P450-dependent mixed function is present in microsomes isolated from human intestine [33] and from CaCo 2 cells [12]; EROD deethylation has been observed in human colon adenocarcinoma at a level equal to that of adjacent normal mucosa [34]. In rats, this activity is five times higher in villous tip than in crypt cells [35]. Nevertheless, the assay of activity on monolayers of living cells makes direct comparison difficult with data obtained with microsomal fractions; the O-deethylation of ethoxyresorufin may however be compared with data reported for cultured hepatocytes in a similar assay [36] and this suggests a level representing about 25% of that reached in liver cells.

Recent reports have indicated that differentiated CaCo 2 cells express glutathione *S*-transferase, with an isozyme composition that most resembles small intestinal cells [11, 12], and perform glucurono- and sulfoconjugation [13, 14]. Nevertheless, under our experimental conditions, resorufin, formed during phase I deethylation by living monolayers of CaCo 2 cells, is excreted in an unconjugated form. This could result either from our culture conditions or, more probably, from the fact that the duration of the incubation and/or of the transit time of the reaction product within the cells was too short to allow the formation of appreciable quantities of conjugates.

O-deethylation activity is increased 50- and 3-fold by methylcholanthrene and phenobarbital, respectively; these substances induce O-de-enzylation but have no effect on depentylation (Table 3). These two inducers do not affect the activity of glutathione *S*-transferase, whereas induction of the liver enzymes has been reported both *in vivo* [37] and *in vitro* [38].

Our results clearly indicate that from 80% to 100% of resorufin (Table 2) and two thirds of glutathione conjugates formed by CaCo 2 cells are excreted at the apical pole (Table 5). This observation could be correlated with recent publications reporting that human intestinal adenocarcinoma cell lines (CaCo 2, HCT-8 and T84) express P-glycoprotein [12, 25] and secrete vectorially vinblastine at their apical pole, in a process sensitive to inhibition by verapamil [25]. In our model, verapamil partially inhibits the excretion of resorufin after O-deethylation; however, this calcium antagonist affects, to the same degree, the amount of resorufin which is recovered at both the apical and basolateral poles. These results could, therefore, be difficult to reconcile with polarized expression of P-glycoprotein at the apical pole and could be better explained by direct inhibition of resorufin formation rather than by impairment of its transport.

The experiments carried out in the presence of verapamil, but also in its absence, indicate the presence of proportions ranging from 0 to 20% of the resorufin formed by the cells in the lower compartment of the bicameral inserts. This could result from direct transport across the basolateral membrane of differentiated CaCo 2 cells, indicating that excretion of this metabolite is not entirely selective. Nevertheless, one cannot exclude transcellular passage of resorufin during the experiment, since our data show a clearance of *ca.* 10%/hr of resorufin to the opposite compartment of the cell culture insert. More precise kinetic data are however required to test this hypothesis.

Finally, these results confirm and extend previous reports from several independent groups indicating that CaCo 2 cells cultivated under conditions favouring the expression of their enterocytic

differentiation biosynthesize intestinal proteins, transport various substances present in the intestinal lumen and are also able to biotransform xenobiotics. This cell culture system provides therefore a good *in vitro* model of the epithelial intestinal barrier. Furthermore, we believe that direct assay of the biotransformation activities on monolayers of living cells allows the combination of transepithelial transport and biotransformation and should better mimic the physiological situation.

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