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Characterization and Inhibition by a Wide Range of Xenobiotics of Organic Anion Excretion by Primary Human Hepatocytes

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ABSTRACT. Organic anion secretion by human hepatocytes was characterized using primary liver parenchymal cell cultures and the anionic fluorescent dye carboxy-2′,7′-dichlorofluorescein (CF). Probenecid, a well-known common blocker of the membrane transport process for anions, was shown to increase CF accumulation in primary human hepatocytes by inhibiting cellular CF efflux in a dose-dependent manner, thereby establishing the presence of an efflux system for organic anions in cultured hepatocytes. Outwardly directed transport of CF from hepatocytes was found to be temperature-dependent; it was not altered by changes in the ionic composition of the incubation medium used in efflux experiments. In addition to probenecid, various structurally and functionally unrelated xenobiotics such as glibenclamide, rifampicin, vinblastine, MK-571, indomethacin, and cyclosporin A were shown to inhibit secretion of CF by primary human hepatocytes, thus suggesting that organic anion excretion by human liver may be impaired by various drugs. Northern blot and Western blot analyses of the expression of multidrug resistance proteins (MRP), such as MRP1 and MRP2, which are known to mediate cellular outwardly directed transport of organic anions indicated that MRP2 was present at substantial levels in cultured human hepatocytes as well as in their *in vivo* counterparts, whereas MRP1 expression was only barely detectable. These results therefore suggest that MRP2, unlike MRP1, may contribute to the organic anion efflux system displayed by primary human hepatocytes and inhibited by a wide range of xenobiotics. BIOCHEM PHARMACOL **60**;12:1967–1975, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. carboxy-2′,7′-dichlorofluorescein; efflux; human hepatocytes; multidrug resistance protein (MRP); primary culture

Hepatic elimination of xenobiotics and endogenous compounds usually involves sequential events including their uptake from the blood flow, their metabolism, and their secretion into the bile. Transport across hepatocyte plasma membranes is therefore a key parameter in their hepatic clearance and commonly occurs through different carrier-mediated systems [1]. Sinusoidal uptake is mediated by distinct transporters such as the organic cation transporter 1 (OCT1) [2], the sodium-independent organic anion transporting polypeptide (OATP) [3], and the sodium-dependent taurocholate co-transporting polypeptide (NTCP) [4]. Similarly, biliary excretion involves several canalicular transporters, in particular ABC† membrane proteins such as P-gp [5], SPGP [6] (also known as the bile salt export pump), and MRP2 [7, 8] (also referred to as the canalicular multispecific organic anion transporter). P-gp usually mediates the secretion of cationic and amphipathic compounds, whereas MRP2 exports anionic compounds, including glutathione and glucuronate conjugates, into the bile [9]. MRP2 belongs to the MRP transporter subfamily that contains at least five other members: MRP1, MRP3, MRP4, MRP5, and MRP6 [10]. MRP1 and MRP3, present in human liver at very low and substantial levels, respectively, share numerous substrates with MRP2, including organic anions [11, 12]. MRP1, MRP2, and MRP3, like P-gp, have also been demonstrated to confer drug resistance on tumoral cells through increased efflux of anticancer drugs [11–13].

While hepatic drug transport processes have been extensively studied in rodents, much fewer data are available in humans and, therefore, there is a need to obtain more experimental data concerning the function and regulation of drug transporters in human liver cells. To this end, human hepatocytes in primary culture have been proposed as a suitable tool [14], especially since *in vivo* experimentation cannot be performed in humans for ethical reasons. Isolated human hepatocytes have thus made it possible to

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† *Abbreviations:* ABC, ATP-binding cassette; CF, carboxy-2′,7′-dichlorofluorescein; CFDA, carboxy-2′,7′-dichlorofluorescein diacetate; DIDS, 4,4′-diisothiocyanostilbene-2,2′disulphonic acid; GB, glutathione bimane; MCB, monochlorobimane; MRP, multidrug resistance protein; P-gp, P-glycoprotein; and SPGP, sister of P-glycoprotein.

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study P-gp-related efflux of cationic drugs [15–17] and membrane inwardly directed transport of biliary acids and organic cations [18]. In the present study, we used primary human hepatocytes to investigate features of hepatic organic anion secretion, including its possible impairment by known inhibitors of ABC transporters. The work was conducted mainly with the anionic dye CF as a prototype of organic anions. Indeed, this fluorescent probe is known to be generated intracellularly by cleavage of its non-polar, non-fluorescent diacetate form (CFDA), which freely diffuses into cells, thus facilitating initial loading of cells [19]. Our results clearly demonstrate the presence of a membrane system involved in CF secretion in primary human hepatocytes. Moreover, this transport system is shown to be inhibited by a wide range of xenobiotics, and we investigated its relationships with some members of the MRP transporter subfamily.

MATERIALS AND METHODS

Chemicals

MCB and CFDA were provided by Molecular Probes. Probenecid, indomethacin, rifampicin, vinblastine, verapamil, glibenclamide, and DIDS were purchased from Sigma Chemical Co. Cyclosporin A and methotrexate were obtained from Sandoz and Wyeth Lerderle Laboratories, respectively. The leukotriene D4 receptor antagonist MK-571 was a generous gift from Dr. Ford-Hutchinson (Merck-Frosst Inc).

Cell Isolation and Culture

Human hepatocytes were prepared by the two-step collagenase perfusion from normal liver fragments of eleven adult donors undergoing resection for primary or secondary tumors. The experimental procedures have been previously described [20]; they complied with French laws and regulations and have been approved by the National Ethical Committee. Freshly isolated hepatocytes were seeded at 37° in a 5% CO_2 atmosphere in Williams' E medium supplemented with bovine insulin (10 μg/mL), 0.2% BSA, and 10% fetal bovine serum. The medium, supplemented with 10^{-7} M dexamethasone but lacking serum, was thereafter renewed daily. Unless otherwise indicated, primary hepatocytes were usually used for dye accumulation and efflux experiments two days after plating. MRP1-overexpressing lung tumoral GLC4/Sb30 cells and parental GLC4 cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine and 10% fetal calf serum as previously described [21].

CF Accumulation Studies

Cells were first rinsed twice with PBS and then incubated with 3 μM CFDA in the presence or absence of 10 mM probenecid in a standard defined incubation medium containing 136 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM D-glucose, and 10 mM HEPES and adjusted to pH 7.4. After 30 min, cells were washed twice with ice-cold PBS and lysed in water by ultrasonication. Amounts of intracellular CF were then evaluated using a Titertek Fluoroscan spectrofluorimeter (Flow Laboratories); excitation and emission wavelengths were 485 and 538 nm, respectively. Results were normalized to cellular protein content determined in parallel using the Bio-Rad assay [22].

CF Efflux Studies

Human hepatocytes were first loaded with 3 μM CFDA in the defined incubation medium described above for 30 min at 37° in the presence of 10 mM probenecid. After washing with ice-cold PBS, cells were re-incubated at 37° in CFDA-free medium for various lengths of time. Intracellular retention of CF was then determined using spectrofluorimetry as reported above. In some experiments, CF-linked fluorescence was also analyzed in aliquots of efflux medium, thus making it possible to directly investigate secretion of CF in the medium.

CF cellular efflux was analyzed under various experimental conditions. CF export was determined in the presence of various drugs added during the efflux period, thus permitting the comparison of CF retention in untreated hepatocytes and drug-treated liver cells. The effects of temperature or ionic composition of the medium were investigated by placing CF-loaded hepatocytes during the efflux phase at 4° or 37° and in various incubation media. These media differed from the standard medium by complete and iso-osmotic replacement of NaCl by KCl or choline chloride, by the use of altered osmotic conditions obtained through changes in NaCl concentrations (hypo-osmotic medium: 68 mM NaCl; hyper-osmotic medium: 272 mM NaCl), or by iso-osmotic replacement of Cl^- in NaCl, KCl, and $CaCl_2$ by gluconate.

GB Efflux Studies

Cells were first loaded with 40 μM MCB for 30 min in the presence of 10 mM probenecid at 37°. The non-fluorescent MCB diffuses into cells where it is conjugated with glutathione, resulting in fluorescent GB [23]. Cells were then washed with ice-cold PBS and re-incubated in MCB-free medium GB at 37° in the presence or absence of 10 mM probenecid for 10 or 20 min. Amounts of GB retained intracellularly or effluxed in the medium were then determined by spectrofluorimetry using a Titertek Fluoroscan spectrofluorimeter; excitation and emission wavelengths were 355 and 460 nm, respectively.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from cells by the guanidium thiocyanate/cesium chloride method of Chirgwin *et al.* [24]. Ten micrograms of total RNAs was subjected to electro-

phoresis in a denaturing 6% (v/v) formaldehyde/1% (w/v) agarose gel and transferred onto Hybond-N^+ sheets (Amersham). The sheets were prehybridized and then hybridized with ^{32}P-labeled probes, washed, dried, and autoradiographed at −80°. Equal RNA loading onto the gel and efficiency of the transfer were checked by methylene blue staining of the membranes and by rehybridizing the blots with an 18S rRNA probe. MRP1, MRP2, and MRP3 mRNAs were detected using human 0.5-kb MRP1, 0.6-kb MRP2, and 1.3-kb MRP3 cDNA fragments, respectively. These probes were generated by reverse transcription–polymerase chain reaction; the specific primers used were: MRP1 sense primer GGACCTGGACTTCGTTCTCA (nucleotides 4109–4128), MRP1 antisense primer GTGTTCCGGATGGTGGACTG (nucleotides 4619–4600); MRP2 sense primer CTAGCAGCCATAGAGCTGGC (nucleotides 269–288), MRP2 antisense primer TGGCTCCAGAGTTCTGCTGG (nucleotides 915–896); MRP3 sense primer CTGCACAGCCTAGACATCCA (nucleotides 2003–2022), MRP3 antisense primer CCTTGGAGAAGCAGTTCAGG (nucleotides 3296–3277). The sequences of the cDNA fragments obtained were determined and found to be similar to those previously published [7, 12, 25].

Protein Isolation and Western Blot Analysis

Total cellular lysates were made by placing harvested cells in a lysing solution containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–Cl pH 7.4, 0.5% SDS, and supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin, and 2 μg/mL of aprotinin. Nuclei were eliminated by a 400-*g* centrifugation. Samples containing 100 μg protein were then fractionated on a 7.5% polyacrylamide gel and then transferred onto nitrocellulose filter by electroblotting. Non-specific binding sites were blocked for 2 hr with Tris-buffered saline containing 10% milk. Monoclonal anti-MRP2 antibodies M_2III-6 [7] (kindly provided by Dr. Scheper, Free University Hospital, Amsterdam, The Netherlands) and monoclonal anti-MRP1 antibodies MRPm6 (Monosan) were then applied to the membranes for 1 hr at room temperature. After washing with Tris-buffered saline containing 0.2% Tween 20, the blot was visualized by chemiluminescence detection using the enhanced chemiluminescence (ECL) system (Amersham).

Statistical Analysis

The results of cellular dye accumulation and efflux studies were processed by Student's *t*-test. The criterion of significance of the difference between the means (± standard deviation) was $P < 0.05$.

RESULTS

Cellular accumulation of CF in primary human hepatocytes was first determined in the presence or absence of 10 mM probenecid. Indeed, this compound is a well-known common inhibitor of membrane transport for anions [26]; therefore, a probenecid-related alteration in cellular accumulation of anionic compounds usually argues for the presence of a carrier-related membrane transport [27]. As indicated in Fig. 1, probenecid was found to increase CF accumulation in primary hepatocytes approximately 2-fold. Similarly, probenecid strongly enhanced cellular CF levels in MRP1-overexpressing GLC4/Sb30 cells, reflecting the elevated expression of an efflux system for anionic compounds in these cells, i.e. MRP1. By contrast, probenecid only weakly augmented CF levels in parental GLC4 cells that display low, although detectable, MRP1 levels [21] (Fig. 1).

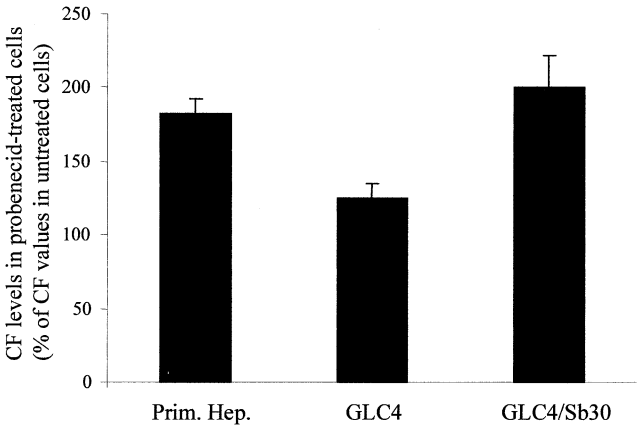

FIG. 1. Effect of probenecid on CF accumulation levels in primary hepatocytes. Two-day-old primary human hepatocytes (Prim. Hep.) and GLC4 and GLC4/Sb30 cells were incubated with 3 μM CFDA in the presence or absence of 10 mM probenecid for 30 min at 37°. Intracellular CF levels were then determined by spectrofluorimetry. The results correspond to cellular CF amounts found in the presence of probenecid expressed as percentages of those obtained in the absence of probenecid. They are the means ± SD of four (primary hepatocytes) or three (GLC4 and CLC4/Sb30 cells) independent experiments in duplicate.

Efflux experiments were next performed to determine whether the action of probenecid was linked to alterations in CF cellular export. As shown in Fig. 2, probenecid (10 mM) was found to strongly limit the loss of cellular CF fluorescence occurring in a time-dependent manner in CF-preloaded hepatocytes studied 48 and 96 hr after plating. Indeed, after a 20-min efflux period, 48-hr-old primary human hepatocytes retained about 20% and 60% of initial dye fluorescence in the absence and presence of probenecid, respectively. In parallel, values of CF measured in the extracellular medium were strongly decreased in response to probenecid (Fig. 3), most likely demonstrating that this compound increased CF retention in primary human hepatocytes by inhibiting dye efflux and not by preventing a putative degradation of CF in non-fluorescent metabolites. This conclusion is also supported by the fact that the sum of intracellular and extracellular CF was similar in primary hepatocytes untreated or treated by probenecid (data not shown), indicating that probenecid did not alter total levels

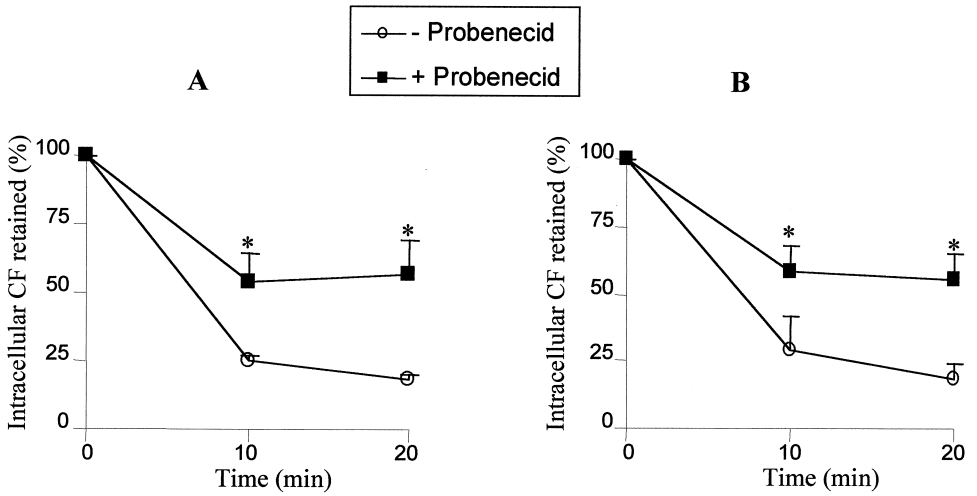

FIG. 2. Effect of probenecid on cellular retention of CF in two-day-old (A) or four-day-old (B) primary hepatocytes. CF-preloaded primary human hepatocytes were incubated in CFDA-free medium for 10 or 20 min at 37° in the presence or absence of 10 mM probenecid. Intracellular CF was then determined by spectrofluorimetry. Values are expressed as percentages of initial staining and are the means ± SD of five independent experiments in duplicate. *, $P < 0.05$ when compared with cells not treated by probenecid.

of CF. The effect of probenecid on CF export from hepatocytes was, moreover, found to be dose-dependent. Indeed, as shown in Table 1, it started at 0.625 mM and was maximal at concentrations ranging from 2.5 to 20 mM.

In order to determine whether probenecid may also alter export from human hepatocytes of anionic compounds distinct from CF, cellular retention of the glutathione *S*-conjugate GB was investigated in efflux experiments performed in the absence or presence of 10 mM probenecid. This compound was shown to increase retention of GB in human primary hepatocytes (Fig. 4A); in parallel, it decreased the amounts of GB secreted into the extracellular medium (Fig. 4B).

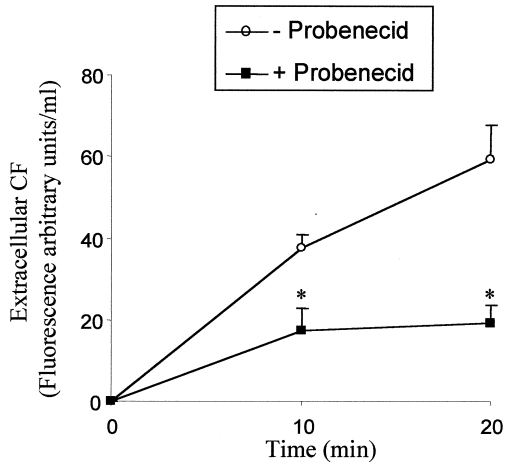

FIG. 3. Effect of probenecid on cellular secretion of CF out of primary hepatocytes. CF-preloaded primary human hepatocytes were incubated in CFDA-free medium for 10 or 20 min at 37° in the presence or absence of 10 mM probenecid. CF released in the medium was then determined by spectrofluorimetry. Values are expressed as fluorescence arbitrary units and are the means ± SD of five independent experiments in duplicate. *, $P < 0.05$ when compared with cells not treated by probenecid.

CF retention levels were then monitored in CF-preloaded primary hepatocytes kept at 4° or 37° over a 20-min efflux period. Hepatocytes maintained at 37° were found to only poorly retain CF-related fluorescence when compared to their counterparts kept at 4° (Fig. 5A). By contrast, extracellular medium from hepatocytes maintained at 37° contained much higher amounts of the fluorescent dye than those detected in medium incubated with hepatocytes kept at 4° (Fig. 5B).

The influence of the ionic composition of the incubation medium on CF efflux from primary human hepatocytes was thereafter studied. As indicated in Fig. 6, the use of an hypo-osmotic (68 mM NaCl) or hyper-osmotic (272 mM NaCl) medium did not modify CF retention. In the same way, complete replacement of NaCl by choline chloride or by KCl did not alter CF staining. The use of gluconate instead of Cl^- was also without obvious effect on CF efflux

TABLE 1. Dose-dependent effect of probenecid on CF retention in primary human hepatocytes

Probenecid (mM)	Intracellular CF retained (%)
0	17.8 ± 2.5
0.156	25.0 ± 6.5
0.312	24.5 ± 5
0.625	32.5 ± 8.4*
1.25	39.6 ± 9.3*
2.5	50.4 ± 9.7*
5	56.9 ± 12*
10	56.4 ± 13*
20	52.0 ± 14*

CF-preloaded primary hepatocytes were incubated in CFDA-free medium for 20 min at 37° in the presence or absence of various doses of probenecid. Intracellular CF retained was then determined by spectrofluorimetry. Values are expressed as percentages of initial staining and are the means ± SD of six independent experiments in duplicate.

*, $P < 0.05$ when compared with cells not treated by probenecid.

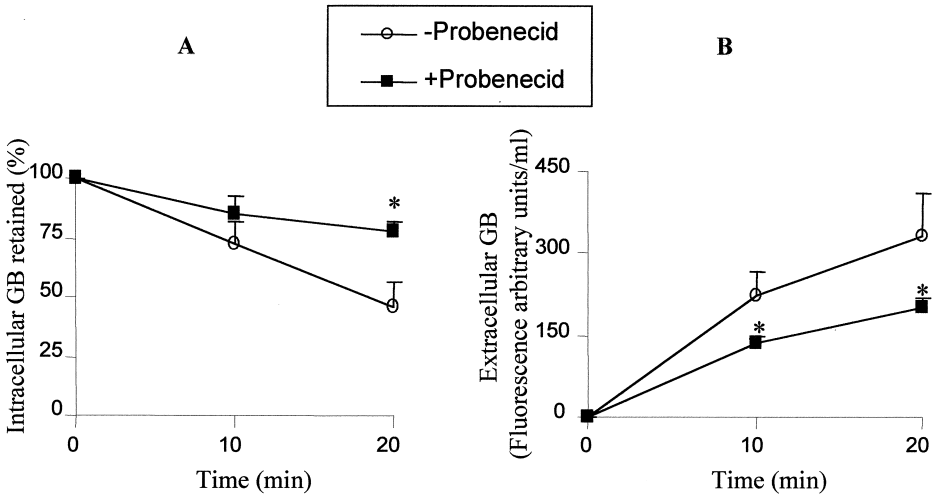

FIG. 4. Effect of probenecid on cellular retention (A) and secretion (B) of GB in primary hepatocytes. GB-preloaded primary human hepatocytes were incubated in MCB-free medium for 10 or 20 min at 37° in the presence or absence of 10 mM probenecid. GB-linked fluorescence retained in the cells (A) or released in the medium (B) was then determined by spectrofluorimetry. Values are expressed as percentages of initial staining (A) or as fluorescence arbitrary units (B) and are the means ± SD of four independent experiments in duplicate. *, $P < 0.05$ when compared with cells not treated by probenecid.

from primary hepatocytes, in contrast to the addition of probenecid (Fig. 6).

In addition to probenecid, several other drugs known to interfere with ABC transporters, such as P-gp and/or MRP1, were tested with respect to their effect on CF efflux displayed by primary human hepatocytes. Glibenclamide, MK-571, indomethacin, rifampicin, vinblastine, and cyclosporin A were found to enhance CF retention in hepatocytes (Table 2) and to decrease the amounts of CF released into the extracellular medium (data not shown). By contrast, verapamil, DIDS, and methotrexate failed to alter cellular CF levels at the concentrations tested (Table 2).

Finally, we investigated expression of MRP1, MRP2, and MRP3 transporters in cultured human hepatocytes by Northern and Western blotting. As shown in Fig. 7, MRP1 mRNA levels were only barely detectable in primary hepatocytes analyzed 48 or 96 hr after their plating. By contrast, very high amounts of MRP1 mRNAs were observed in GLC4/Sb30 cells used here as positive control. Parental drug-sensitive GLC4 cells were also found to express MRP1 mRNAs at a level much lower than that detected in GLC4/Sb30 cells, but greater than that observed in primary human hepatocytes (Fig. 7). In contrast to MRP1 mRNAs, those of MRP2 and MRP3 were clearly apparent in cultured human hepatocytes, whereas they were not obviously detected in either GLC4 or GLC4/Sb30 cells (Fig. 7). Similarly, SPGP mRNAs were detected in primary human hepatocytes but not in GLC4 and GLC4/Sb30 cells as assessed by reverse transcriptase–polymerase chain reaction analysis (data not shown). Western blotting indicated

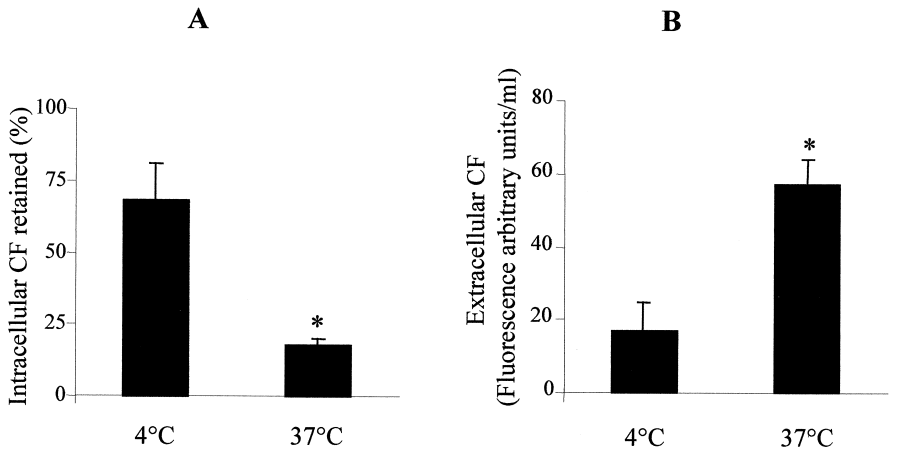

FIG. 5. Effect of temperature on cellular retention (A) and secretion (B) of CF in primary human hepatocytes. CF-preloaded primary human hepatocytes were incubated in CFDA-free medium for 20 min at 4° or 37°. CF-linked fluorescence retained in the cells (A) or released in the medium (B) was then determined by spectrofluorimetry. Values are expressed as percentages of initial staining (A) or as fluorescence arbitrary units (B) and are the means ± SD of four independent experiments in duplicate. *, $P < 0.05$ when compared with cells incubated at 4°.

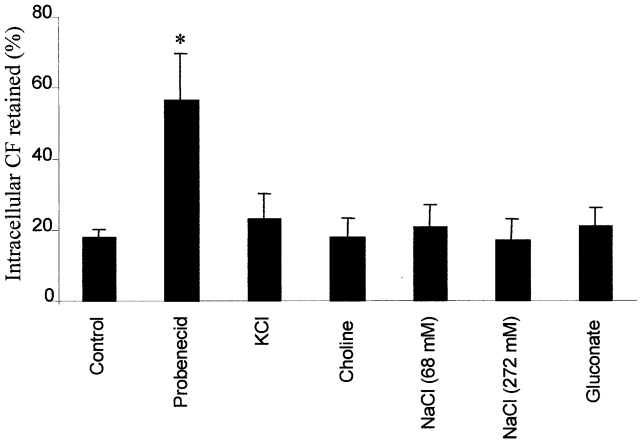

FIG. 6. Effect of ionic composition of the incubation medium on CF retention in primary human hepatocytes. CF-preloaded primary hepatocytes were placed for 20 min at 37° in media differing from control medium by the addition of 10 mM probenecid, by complete iso-osmotic substitution of NaCl by choline chloride or KCl, by the use of altered concentrations of NaCl (68 or 272 mM), or by iso-osmotic replacement of Cl^- by gluconate in NaCl, KCl, and $CaCl_2$. CF-linked fluorescence retained in the cells was then determined by spectrofluorimetry. Values are expressed as percentages of initial staining and are the means of at least five independent experiments in duplicate. *, $P < 0.05$ when compared with control medium.

the presence of a 190-kDa M_2 III-6 antibody-reactive band corresponding to MRP2 in primary human hepatocytes as in freshly isolated human hepatocytes and in human liver (Fig. 8); this band was not detected in GLC4 and GLC4/Sb30 cells. By contrast, Western blotting clearly showed a 190-kDa band corresponding to MRP1 in GLC4/Sb30 cells and also, although to a much lesser extent, in parental GLC4 cells, whereas such a MRP1-related band was not, or only very faintly, detected in primary human hepatocytes in freshly isolated human hepatocytes and in human liver (Fig. 8).

TABLE 2. Effect of various drugs on CF retention in primary human hepatocytes

Drug	Concentration	Intracellular CF retained (%)
None	—	17.8 ± 2.5
Probenecid	10mM	56.4 ± 13*
Verapamil	100 μM	13.4 ± 2.4
Glibenclamide	100 μM	55.4 ± 11*
MK-571	50 μM	54.1 ± 10*
Indomethacin	200 μM	54 ± 12*
Rifampicin	200 μM	47.5 ± 11*
DIDS	100 μM	21.5 ± 9.5
Vinblastine	100 μM	42.5 ± 9.9*
Cyclosporin A	10 μM	57.2 ± 16.1*
Methotrexate	100 μM	20.4 ± 4.7

CF-preloaded primary hepatocytes were incubated in CFDA-free medium for 20 min at 37° in the presence or absence of various drugs. Intracellular CF retained was then determined by spectrofluorimetry. Values are expressed as percentages of initial staining and are the means ± SD of at least five independent experiments in duplicate.

*, $P < 0.05$ when compared with control cells.

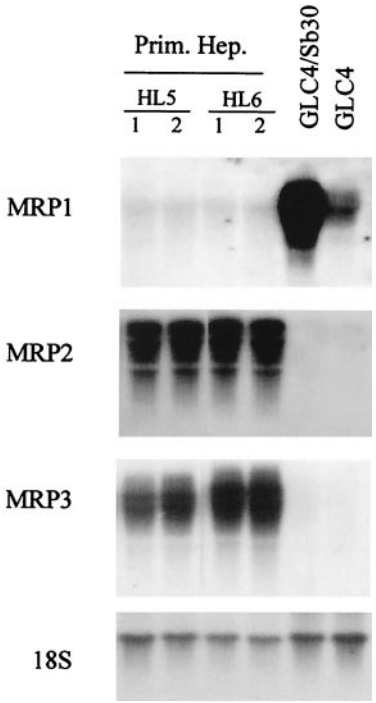

FIG. 7. Expression of MRP1, MRP2, and MRP3 mRNAs in primary human hepatocytes. Each lane contains 10 μg total RNA isolated from primary human hepatocytes (Prim. Hep.) 48 hr (1) or 96 hr (2) after their plating or from GLC4 and GLC4/Sb30 cells. RNA were transferred onto Hybond-N^+ sheets after electrophoresis and hybridized with MRP1, MRP2, MRP3, and 18S probes. The results shown were obtained with primary hepatocyte cultures prepared from two human livers (HL5 and HL6); similar data were also observed with hepatocytes from three additional livers.

DISCUSSION

This study was designed to investigate features of hepatic organic anion secretion using primary human hepatocytes. The data shown indicate that cultured human hepatocytes are able to excrete organic anions as do their *in vivo* counterparts. Indeed, intracellular accumulation of the anionic dye CF in the cultured parenchymal liver cells was found to be strongly enhanced by probenecid, a common blocker of organic anion transporters [26], and such an effect was demonstrated, through efflux experiments, to be linked to dose-dependent inhibition of cellular extrusion of the dye by probenecid. In addition, probenecid was demonstrated to inhibit secretion of GB by primary human hepatocytes. A probenecid-inhibitable efflux system for organic anions, including glutathione conjugates such as GB, is therefore constitutively present in primary human hepatocytes and likely at substantial levels, since the effect of probenecid on cellular CF accumulation was similar in the cultured liver cells and in GLC4/Sb30 cells that markedly express an MRP1-related efflux system for anionic compounds [21]. In addition, the CF export system found in primary human hepatocytes was well preserved during the period of culture, since similar low levels of retention of the dye were found in hepatocytes 48 and 96 hr after their plating.

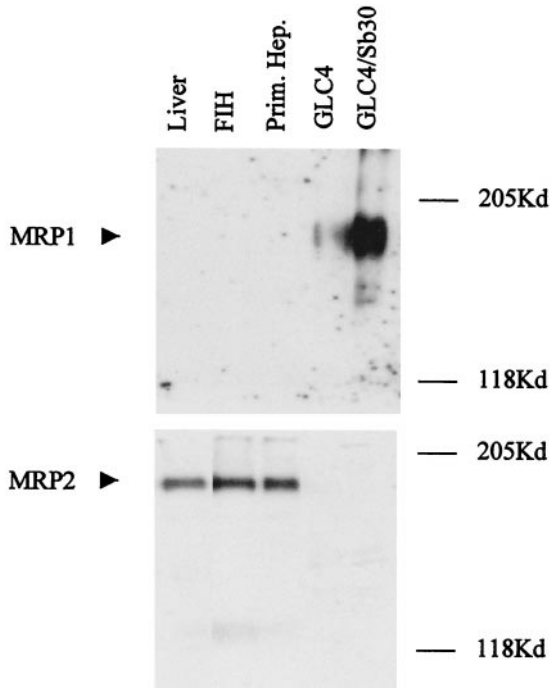

FIG. 8. Expression of MRP1 and MRP2 in primary human hepatocytes. Cell lysate samples (100 μg protein) prepared from human liver, freshly isolated hepatocytes (FIH), two-day-old primary hepatocytes (Prim. Hep.), and GLC4 and GLC4/Sb30 cells were separated on polyacrylamide gels and transferred onto nitrocellulose sheets. After incubation with monoclonal antibodies raised against MRP1 and MRP2, the blots were developed using an enhanced chemiluminescence kit. The position of molecular mass standards in kDa are indicated on the right. The data shown, obtained with primary hepatocytes prepared from one human liver, are representative of the analysis of cultured hepatocytes originated from three other livers.

Such an outwardly directed transport system for organic anions was shown to be temperature-dependent, since CF efflux was strongly decreased when human hepatocytes were placed at 4° instead of 37°. Organic anion secretion by human hepatocytes, then, is likely an active process requiring energy, as already demonstrated for the efflux of anionic compounds from rat hepatocytes [28].

Cellular CF efflux from human hepatocytes was not altered by iso-osmotic replacement of NaCl by choline chloride in the incubation medium, demonstrating that it was not directly driven by a Na^+ gradient. Similarly, substitution of Cl^- by gluconate in NaCl, KCl, and $CaCl_2$ did not affect cellular CF retention, indicating that CF transport was not dependent on a Cl^- gradient. In the same way, iso-osmotic replacement of NaCl by KCl, which has been shown to result in dissipation of the plasma membrane potential of isolated hepatocytes as measured by the equilibrium distribution of $^{36}Cl^-$ [29], was without effect on CF secretion from primary human hepatocytes. Organic anion excretion from human hepatocytes is thus not linked to plasma membrane potential and therefore may be an electroneutral transport. Similarly, outwardly directed transport of organic anions in rat hepatocytes has been shown not to be altered by a change in membrane potential [29]. The use of hypo- or hyper-osmotic incubation medium during the efflux phase also did not alter CF efflux from primary human hepatocytes, likely indicating that manipulation of medium osmolarity does not directly influence organic anion secretion in liver cells. Such a conclusion is also supported, at least for hypo-osmotic conditions, by the fact that hypo-osmotic exposure has no effect on the steady-state excretion of bromosulphtalein into bile in isolated perfused liver experiments [30].

In addition to probenecid, various structurally and functionally unrelated xenobiotics were found to inhibit CF efflux from human hepatocytes. Indeed, the sulfonylurea glibenclamide, the antituberculosis compound rifampicin, the anticancer drug vinblastine, the leukotriene D4 receptor antagonist MK-571, the non-steroidal anti-inflammatory agent indomethacin, and the immunosuppressive agent cyclosporin A were demonstrated to increase CF retention in primary human hepatocytes. Interestingly, most of these compounds, if not all, have already been shown to block activity of drug transporters, especially that of ABC proteins. Probenecid, indomethacin, and MK-571 thus inhibit MRP1-mediated transport [31–33], whereas cyclosporin A, vinblastine, and glibenclamide down-regulate P-gp-related drug efflux [34, 35]. Taken together, these results therefore highlight the lack of specificity of some inhibitors of drug transport. This conclusion is further supported by the fact that some compounds initially described as P-gp modulators, such as rifampicin or the antiprogestatin compound RU 486, also block MRP1 activity in drug-resistant tumoral cells [36, 37] and, at least for rifampicin, CF efflux from human hepatocytes. Such an absence of specificity cannot, however, be considered as a general rule. Indeed, verapamil, a strong inhibitor of P-gp function [34], did not affect CF secretion from primary human hepatocytes and only marginally affects MRP1 activity [11]. In the same way, DIDS, a well-known blocker of anion membrane transport [38], did not alter CF efflux in human liver cells. The anticancer drug methotrexate, which has recently been demonstrated to be handled by MRP1 and MRP2 [39], was also found not to interfere with CF transport at the concentration tested. It should, however, be kept in mind that the absence of inhibition by a compound does not necessarily mean that such a compound is not a substrate for the transporter studied [40].

The nature of the membrane carrier responsible for the efflux of organic anions such as CF from primary human hepatocytes remains to be determined. Involvement of MRP1, which has been shown to handle CF in cultured biliary epithelial cells [19] and which is inhibited by most of the compounds blocking CF secretion from primary human hepatocytes, seems to be very unlikely. Indeed, as in human liver, MRP1 expression was only barely detectable in cultured human hepatocytes. By contrast, MRP1, unlike other MRP transporters such as MRP2 and MRP3, was present at a very high level in GLC4/Sb30 cells and therefore likely handled CF in such cells as already suggested [41]. A major contributing transporter to CF secre-

tion by primary human hepatocytes may be MRP2, since this protein was found to be highly expressed in cultured human hepatocytes, as in human liver, as demonstrated by Northern and Western blotting. Moreover, hepatocytes from TR^- mutant Wistar rats lacking MRP2 expression failed to secrete CF, in contrast to their counterparts obtained from control rats, therefore indicating that CF is a substrate for rat MRP2 [42]. In addition, some of the compounds, such as cyclosporin A, that inhibited CF efflux from human hepatocytes have already been demonstrated to block the activity of MRP2 in rat liver cells [43]. A contribution of other transporters of organic anions, including as yet unidentified or recently described ABC transporters, to CF secretion by primary human hepatocytes cannot, however, be discarded. In this context, MRP3, found to be expressed at substantial levels in cultured human hepatocytes as assessed by Northern blotting, likely does not participate in CF efflux, since MRP3-overexpressing hepatocytes from TR^- rats have been shown not to secrete the dye [42] and since MRP3 activity has been reported to be blocked by methotrexate [44], a compound ineffective on CF secretion by primary hepatocytes. Similarly, SPGP, although detected in primary hepatocytes, probably does not contribute to CF transport since SPGP activity has been postulated to be highly inhibited by verapamil [45], which failed to block CF export from hepatocytes. Involvement of MRP5 is also unlikely since this transporter, present at very low levels in the liver, is not blocked by compounds inhibiting CF secretion from human hepatocytes, such as probenecid [46].

In summary, organic anion secretion by human liver parenchymal cells was evidenced and characterized using primary hepatocytes. Such an outwardly directed transport likely involved activity of MRP proteins such as MRP2; it was found to be decreased by a wide range of xenobiotics, suggesting potential drug interactions with respect to biliary secretion of anionic drugs or glutathione conjugates. Moreover, the presence of an organic anion transport system in primary human hepatocytes and also that of the cationic drug transporter P-gp [15] suggests that such cells, in addition to metabolizing drugs [14], are able to excrete them or their metabolites as do their *in vivo* counterparts. These data therefore outline the interest of primary cultures of human hepatocytes in the investigation of drug-detoxifying pathways.

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