Unconjugated Bile Acids Modulate Adult and Neonatal Neutrophil Chemotaxis Induced *In Vitro* by *N*-Formyl-Met-Leu-Phe-Peptide

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ABSTRACT

In this study, we have investigated the effect of hydrophobic and hydrophilic unconjugated bile acids (UBAs)—ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), lithocholic acid, and colic acid—on chemotaxis in adult and neonatal human polymorphonuclear leukocytes (PMNs). The trypan blue exclusion dye test was preliminarily performed to determine the toxicity of the studied UBAs on PMNs. N-formyl-methionyl-leucyl-phenylalanine (100 nM) was used as a chemoattractant. Chemotaxis (1×10^6 cells/mL) was analyzed in the presence or absence of UBAs (10 μM) by blind well chambers. The antioxidants vitamin E and vitamin C were tested for their ability to reduce the inhibitory effect of UBAs. We found that only CDCA was able to induce damage in PMNs in the range of 1–40 μM. Both CDCA and UDCA were able to inhibit chemotaxis in PMNs, whereas lithocholic acid and colic acid were ineffective. The inhibitory effect was reversible inasmuch as PMNs incubated with either CDCA or UDCA and subsequently washed showed normal chemotaxis. Concomitant incubation of PMNs with UBAs and vitamins C or E reversed the inhibition. We did not find substantial differences between PMNs from adults or newborns. In conclusion, CDCA and UDCA are able to reduce, in a specific and reversible fashion, both adult and newborn neutrophil chemotaxis. As concomitant incubation of UBAs and electron scavengers restores PMN chemotaxis to control values, we conclude that free radicals may be involved in the mechanism of inhibition. We speculate that this defect may contribute to the impaired host response described in cholestatic patient. (*Pediatr Res* 51: 392–396, 2002)

Abbreviations
PMN, polymorphonuclear leukocytes
UBA, unconjugated bile acid
UDCA, ursodeoxycholic acid
CDCA, chenodeoxycholic acid
LTCA, lithocholic acid
CA, colic acid
fMLP, N-formyl-methionyl-leucyl-phenylalanine

The high incidence of postsurgical septic complications in patients with obstructive jaundice (1–3) and the wide use of UDCA in human therapy (4–6) have prompted previous investigators to study the effect of both bile as a whole and UBAs on PMN function. However, conflicting results have been obtained. In surgical models of cholestasis in the rat, both depressed (7, 8) and enhanced (9) PMN functions have been described. Recently, D'Odorico *et al.* (10) reported no altered production of reactive oxygen metabolites in neutrophils from patient with chronic cholestatic liver disease. In contrast, others (11) described increased hydrogen peroxide and superoxide anion generation from patients with obstructive jaundice. These differences may be explained by different experimental conditions and/or by the experimental models used, *e.g.* the profound differences that exist between bile acid metabolism in rats and humans (12). Another cause of bias may be the peculiar ability of PMNs to easily undergo activation or to be primed, conditions that may occur during surgical manipulation of rats.

Only one study, to our knowledge, has previously investigated the effect of bile as a whole on PMN chemotaxis (9), whereas Chen *et al.* (13) recently described the ability of CDCA to act as an antagonist of the formyl-peptide receptor in human monocytes and in transfected cells. However, these authors used bile acids at high concentrations and did not provide any information about their toxicity on human neutrophils.

The high incidence of hepatobiliary disease in newborns that have defective PMN function has been observed (14–16), however, the effect of UBAs on the natural immune systems of neonates has not been investigated to date.

The aim of our work was to study the effect of individual UBAs on both adult and newborn human PMN chemotaxis in an *in vitro* model. We report the effect of UBAs differing in their hydrophilic/hydrophobic properties: from the relatively hydrophilic CA (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid), to CDCA (3α, 7α-dihydroxy-5β-cholan-24-oic acid) and its 7β-epimer UDCA (widely used in bile acid therapy), to the most hydrophobic, LTCA (3α-hydroxy, 5β-cholan-24-oic acid).

MATERIAL AND METHODS

Chemicals. All culture media were purchased as sterile and endotoxin tested solutions from Sigma Chemical (St. Louis, MO, U.S.A.).

UDCA, CDCA, LTCA, CA, fMLP, vitamin C, vitamin E, Ficoll-Hypaque, and Dextran were also obtained from Sigma Chemical. Other products were of analytical reagent grade. Bile acids (500 mM) were dissolved in DMSO and stored at $4°C$ for no longer than a week. Working solutions were prepared just before use in RPMI media.

Because of the low solubility of vitamin E in water, buffers, DMSO, and similar partially polar solvents, and because of the ability of ethanol to interfere with PMN functions (17), we dissolved the vitamins in diethyl ether. The exact amount of diethyl ether dissolved vitamin E required for each experiment was pipetted into the appropriate vial. The solvent was evaporated in a vacuum overnight under agitation. Then, one half of the final volume of tissue culture media was added and the vials were vortexed extensively. Lastly, we added cells or UBAs to give the final concentrations mentioned in the text. Control samples were prepared accordingly.

Sampling of blood. The local ethical committee approved this study. Mothers gave consent for cord blood collection before delivery. Similarly, adult volunteers gave their consent for blood drawing.

Heparin at a concentration of 20 U/mL was used as anticoagulant. Venous blood was collected from the placental vessels of full-term infants who were delivered vaginally. Newborns were the product of uncomplicated pregnancies and deliveries; neither general nor local anesthesia was administered to the mothers. No perinatal complications occurred in any of the babies studied, and all had an Apgar score ≥ 8 at 1 and 5 min. Blood from healthy adult donors was drawn from the forearm vein and studied simultaneously with cord blood samples.

PMN purification. Immediately after blood collection, blood was layered over Ficoll-Hypaque (density = 1.077) and centrifuged at $400\times g$ for 30 min to remove mononuclear cells.

Erythrocytes were separated by 2% dextran (M_r 480,000) sedimentation for 30 min at room temperature. Residual erythrocytes were removed by hypotonic lysis with cold 0.2% NaCl for exactly 30 s. Adding an equal volume of cold 1.6% NaCl restored isotonicity.

Cells were washed twice and suspended in RPMI containing 5% FCS. PMNs obtained by this technique were $\geq 97\%$ pure and $\geq 99\%$ viable.

Trypan blue exclusion dye test. Trypan blue exclusion dye test was used to determine the toxicity of UBAs. Cells were incubated for 45 min ($37°C$, 5% CO_2, 95% relative humidity) at various UBA concentrations in the presence of 0.2% trypan blue. Stained PMNs were counted at a magnification of $\times 400$.

Chemotaxis. fMLP (100 nM) was used as a chemoattractant. This was the minimal concentration required to stimulate the maximal chemotactic response from human neutrophils (not shown). Cells (1×10^6/mL) were loaded in the upper well of blind chambers (200 μL, NeuroProbe, Gaithersburg, MD, U.S.A.) equipped with 3-μm polycarbonate filters. DMSO-dissolved UBA was added to PMN suspensions at a final concentration of 10 μM (DMSO $<0.01\%$). After the appropriate incubation period (30 min at $37°C$, 5% CO_2, 95% relative humidity) filters were removed and fixed by immersion in methanol. Cells were stained with 10% Giemsa solution. The cells that migrated through the pores were counted at 1000-fold magnification from four randomly selected fields. PMNs that dropped off the membrane were recovered from the lower chamber, counted, and added to the total number of migrated cells.

Reversibility of the inhibition induced by CDCA and UDCA. The appropriate bile acid was added to the PMN suspension (in these experiments, the cell concentration was 2 $\times 10^6$/mL to compensate for the loss of cells that occurs during the following centrifugation steps) and incubated for 20 min at $37°C$. Cells were then washed three times with the culture media and suspended to the initial volume. Chemotaxis was performed as already described.

Control tubes were prepared as follows: PMN suspensions (2×10^6/mL) were incubated with the solvent alone for 20 min at $37°C$, washed three times with the culture media, and then the appropriate bile acid (or the solvent alone) was added. Chemotaxis was performed as above.

We also tested the ability of the antioxidant vitamins E and C to revert the inhibitory effect of CDCA or UDCA as an indirect evidence of involvement of free radicals in the mechanism of chemotaxis inhibition (18, 19). In these experiments, the vitamins were added concomitantly with the UBAs, without any preincubation.

Statistical analysis. The significance of differences between the groups was tested by the one-way ANOVA. We used Tukey's post hoc test to compare pairs of group means. Results are reported as mean $\pm$ SD.

RESULTS

Cell viability. Cell viability was studied at UBA concentrations ranging from 1 to 40 μM. As shown in Figure 1, only CDCA demonstrated a clear dose-response cytotoxicity for human PMNs. In the presence of 5 μM CDCA, trypan blue–positive cells increased from $0.53 \pm 0.28\%$ (control) to $5.7 \pm 1.2\%$ for the adult PMNs and from $0.8 \pm 0.91\%$ (control) to $4.8 \pm 0.65\%$ for the cord blood PMNs (mean $\pm$ SD; $p < 0.01$, $n = 4$). At the maximal concentration tested (40 μM), CDCA-induced cell death increased up to $21.98 \pm 0.75\%$ and $24 \pm 3.00\%$ in PMNs from both adults and neonates, respectively. The other UBAs did not significantly affect cell viability except for a slight but not significant ($p > 0.05$, $n = 4$) increment of trypan blue–positive cells observed in the presence of 40 μM
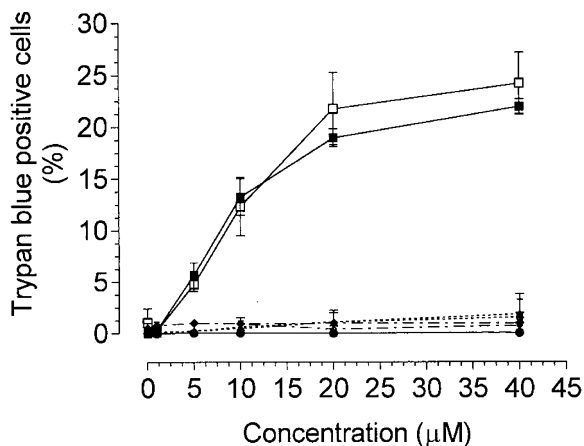
Figure 1. Cell damage induced by bile acids in human PMNs. Only CDCA was able to induce significant cell injury and consequent staining of PMNs by the trypan blue exclusion dye test. The presence of the other bile acids did not significantly affect cell viability. Experiments performed with adult PMNs: *filled squares* = CDCA; *filled triangles* = UDCA; *filled circles* = LTCA; *filled diamonds* = CA. Experiments performed with neonatal PMNs: *open squares* = CDCA; *open triangles* = UDCA; *open circles* = LTCA; *open diamonds* = CA.

UDCA. We did not find significant differences between UBA toxicity in adult or cord blood PMNs.

Inhibition of fMLP-induced PMN locomotion. Both CDCA and UDCA (10 μM) were able to inhibit significantly fMLP-induced chemotaxis ($p < 0.01$, $n = 6$). The effect was similar in adult and neonatal samples. Neither LTCA nor CA inhibited cell locomotion significantly ($p > 0.05$, $n = 6$). Results are summarized in Figure 2.

As reported, as the concentration of CDCA and UDCA increases, the chemotaxis, expressed as a percentage of the maximal response in the absence of inhibitor, decreases. Concentrations of 20 and 40 μM resulted in the inhibition of the random PMN motility.

It should be pointed out that at the concentrations of 1 and 5 μM, UDCA, despite the lower toxicity, was more effective than CDCA (33.22 $\pm$ 2.26% *versus* 87.71 $\pm$ 0.90%; 30.29 $\pm$ 2.72% *versus* 46.01 $\pm$ 2.47% for the adults and 34.67 $\pm$ 9.484% *versus* 84.79 $\pm$ 5.643%; 36.15 $\pm$ 4.186% *versus* 45.54 $\pm$ 3.339% in neonatal PMNs, respectively; $p < 0.001$, $n = 6$).

Chemotaxis inhibition is reported as a percentage of the maximal response against a control chemotaxis. This allows a direct comparison between adult and neonatal samples. However, as already reported, neonatal PMNs have a natural decreased motility (14).

Reversibility of chemotaxis inhibition. Human PMNs incubated with either CDCA or UDCA and subsequently washed showed a chemotactic response not significantly different from control fMLP-induced chemotaxis (for CDCA and UDCA: 110.70 $\pm$ 18.04% and 104.30 $\pm$ 5.32% for the adult; and 105 $\pm$ 16.4% and 110.50 $\pm$ 17.0% for neonatal PMNs, respectively). These results were from six separate experiments.

Effect of vitamin C and vitamin E on bile acid–induced chemotaxis inhibition. Both vitamins were capable of wholly restoring cell locomotion in a dose-dependent manner. In
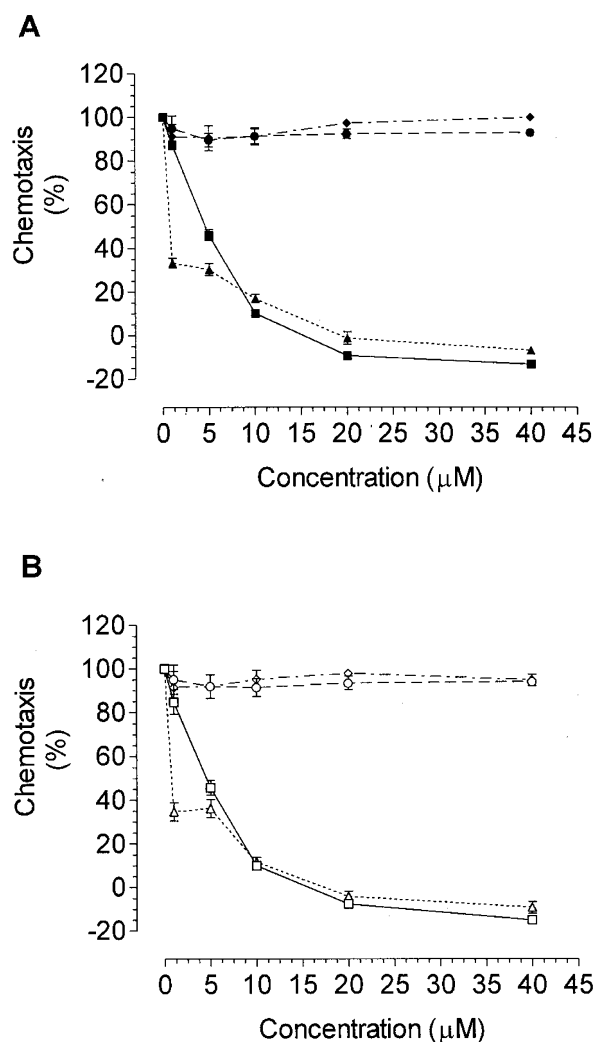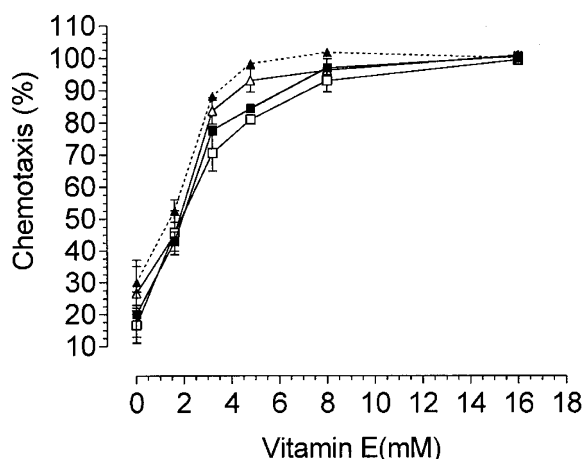

Figure 2. Effect of bile acids on fMLP-induced chemotaxis. Both CDCA and UDCA were able to reduce significantly the fMLP-induced chemotaxis in both adult (*panel A*) and neonatal samples (*panel B*). Similar results were obtained from adult and neonatal PMNs. Results were normalized assuming control = 0 and fMLP-stimulated chemotaxis = 100. Experiments performed with adult PMNs: *filled squares* = CDCA; *filled triangles* = UDCA; *filled circles* = LTCA; *filled diamonds* = CA. Experiments performed with neonatal PMNs: *open squares* = CDCA; *open triangles* = UDCA; *open circles* = LTCA; *open diamonds* = CA.

particular, 100 μM vitamin C could reverse the inhibitory effect of UBAs on fMLP-induced chemotaxis (Fig. 3).

Vitamin E had similar effects on human PMNs, but milli-molar concentrations were needed to restore the chemotactic response (Fig. 4). This may the result of the low solubility of vitamin E in aqueous solutions. For both vitamins, similar results were obtained in adult and neonatal PMNs.

DISCUSSION

In this study, we have tested individual UBAs in an *in vitro* system to determine their ability to impair PMN chemotaxis. The concentrations used are relevant to most clinical situations. In fact, normal serum concentrations of each specific UBA range between 0.2 and 1 μM (20). However, they may increase up to 150 μM in chronic cholestatic disease (21). Moreover,

Figure 3. Dose-dependent effect of vitamin C on fMLP-induced chemotaxis inhibited by either CDCA or UDCA (10 μM). Vitamin C is capable of reversing the inhibitory effect of bile acids on fMLP-induced chemotaxis at a concentration of 100 μM in both adult and neonatal samples. Results were from three separate experiments reported as normalized, assuming control = 0 and the fMLP-stimulated chemotaxis = 100. Experiments performed with adult PMNs: *filled squares* = CDCA; *filled triangles* = UDCA. Experiments performed with neonatal PMNs: *open squares* = CDCA; *open triangles* = UDCA.
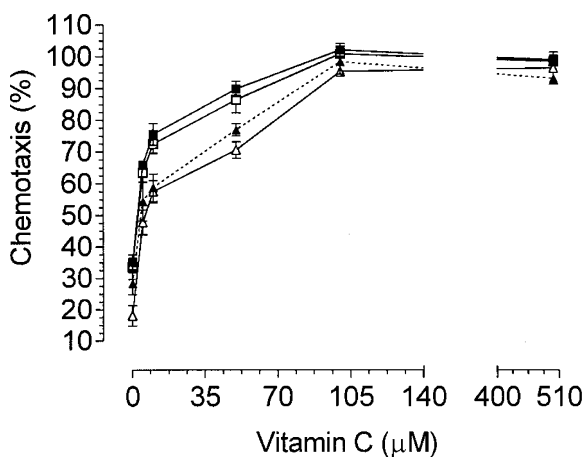
Figure 4. Dose-dependent effect of vitamin E on fMLP-induced chemotaxis inhibited by either CDCA or UDCA (10 μM). Vitamin E is capable of reversing the inhibitory effect of bile acids on fMLP-induced chemotaxis at a concentration of 8 μM. Similar results were obtained from adult and neonatal samples. Results were from three separate experiments reported as normalized, assuming control = 0 and the fMLP-stimulated chemotaxis = 100. Experiments performed with adult PMNs: *filled squares* = CDCA; *filled triangles* = UDCA. Experiments performed with neonatal PMNs: *open squares* = CDCA; *open triangles* = UDCA.

tissue concentrations of about 250 μM are common in cholestasis (22).

Interestingly, only two bile acids—CDCA and its β-epimer UDCA—decrease, in a dose-dependent fashion, neutrophil recruitment, whereas both the more hydrophilic CA and the hydrophobic LTCA (23) were not effective. This inhibitory effect does not involve strong chemical bonds inasmuch as it is fully reverted by a simple washing procedure. Furthermore, the functional effect of UBAs appears unrelated to cytotoxicity. In fact, UDCA, although effective in regulating PMN locomotion, is significantly less toxic than the structurally similar CDCA

(Fig. 1). Intriguingly, functional impairment by CDCA and UDCA is fully reversed by co-incubation with both vitamins C and E, which may act as free radical scavengers. These data, taken together, demonstrate a specific UBA-cell interaction other than the detergent effect so often claimed to explain the many biologic effects of UBAs.

It has been previously demonstrated that high CDCA concentrations (100–200 μM) cause irreversible cell damage using oxygen free radicals as intracellular messengers in rat hepatocytes (18, 24) and cultured macrophages (25). Our data, though, show that substantially lower concentrations of both CDCA and UDCA are able to functionally impair in a reversible fashion the human PMN chemotaxis. Evidence from the experiments with antioxidant vitamins suggests that there is an intimate interplay between UBAs and free radicals in PMN locomotion, the mechanisms of which are currently being studied.

Our findings show that UDCA and CDCA are able to modulate the chemotaxis of both adult and neonatal PMNs. Thus, as neonates have a chemotactic motility significantly lower than the adults (14), the down-modulation induced by either UDCA or CDCA may further aggravate the natural impairment of neonatal PMNs.

In conclusion, we provide evidence that CDCA and UDCA modulate human PMNs chemotaxis *in vitro*. These observations may help explain both the complex immune derangement of the cholestatic patient and the immunomodulating potential of UDCA that has gained a wide therapeutic success.

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