p-Cresol, a Uremic Compound, Enhances the Uptake of Aluminum in Hepatocytes

KENNETH ABREO,* M’LISS SELLA,* SCOTT GAUTREAUX,* RITA DE SMET,† PASCALE VOGELERE,† SEVERIN RINGOIR,† and RAYMOND VANHOLDER†
*Department of Medicine, Louisiana State University School of Medicine, Shreveport, Louisiana; and †University Hospital, Ghent, Belgium.

Abstract. In the end-stage renal disease patient, certain uremic compounds could influence the cellular accumulation of aluminum (Al). In this study, we examined the effect of 15 uremic ultrafiltrate fractions obtained by HPLC on the uptake and toxicity of Al in mouse hepatocytes (MH) in culture, a model system in which Al is taken up bound to transferrin (Tf). Uremic fractions 4 to 8, 12, 14, and 15 increased cellular Al uptake and aspartate aminotransferase release and decreased cell growth when Tf-Al, not Al citrate, was added to culture media. Compounds that have been extracted previously from these ultrafiltrate fractions (p-cresol, xanthine, tryptophan, hippuric acid, and α-hydroxyhippuric acid) were then tested for their effect on Al uptake and toxicity in MH at concentrations found in uremic serum. Significant Al uptake by MH was observed only when p-cresol was added together with Tf-Al. Time–response curves showed increased Al uptake and toxicity at p-cresol concentrations of 3 mg/dl in culture media. Dose–response curves confirmed that Al uptake and cell toxicity were proportional to p-cresol from 1.5 mg/dl to 3 mg/dl in culture media. p-Cresol was not toxic to MH in the absence of Tf-Al in media. p-Cresol increased Tf-associated Al uptake only because there was no effect on Al uptake when Al citrate was substituted, and studies with Tf-I125-Al in the presence of this compound showed increased Tf-I125 taken up by MH. p-Cresol did not increase Tf saturation with Al. p-Cresol also increased Tf-Al uptake in Friend erythroleukemia and neuroblastoma cells in culture. Our studies suggest that p-cresol and uremic fractions 4 to 8, 12, 14, and 15 increase the uptake and toxicity of Al in cultured MH. These compounds may play a role in the accumulation and toxicity of Al in the liver of end-stage renal disease patients and possibly in all cells that express Tf receptors. (J Am Soc Nephrol 8: 935–942, 1997)

Aluminum (Al) accumulation in end-stage renal disease (ESRD) patients can occur from exposure to contaminated dialysate or the ingestion of Al-containing antacids and can result in encephalopathy, bone disease, and anemia (1, 2). Certain subsets of ESRD patients, such as the very young or old, diabetic and postparathyroidectomy patients, and those who have recently lost a renal transplant, are most susceptible to Al overload, suggesting that the uremic milieu in these patients may favor Al accumulation and toxicity (3, 4). Although the effect of uremia on the gastrointestinal absorption and organ deposition of Al in ESRD patients is not known, animal studies suggest that uremia favors the absorption and deposition of Al (5). Because increased parathyroid hormone and decreased 1,25 vitamin D levels are typically seen in renal failure, the effect of these hormones on Al absorption and deposition was initially evaluated. Initial studies in rats administered Al compounds suggested that parathyroid hormone enhanced the gastrointestinal absorption of Al (6), and 1,25 vitamin D decreased the organ deposition of Al (7); however, recent studies suggest that these hormones do not play a significant role (8). An alternative hypothesis to explain the increased susceptibility of uremic animals to Al toxicity is that the accumulation of certain poorly dialyzed compounds may enhance the absorption and deposition of Al.

Because both the absorption of Al in the gastrointestinal tract (9) and its subsequent deposition in organs involve its cellular uptake, we evaluated the effect of uremic compounds on cellular Al uptake. A major fraction of Al is sequestered in the liver in man and in animals with Al overload (10, 11). Al administration in rats and piglets has resulted in elevation of bile acid concentrations, decreased bile flow, and suppression of cytochrome P-450 (12–14). Although gross hepatic toxicity has not been reported in dialysis patients with Al excess, cholestatic liver disease from Al has been reported in children receiving parenteral nutrition (15). It is possible that the toxic effects of Al on the adult liver are too subtle to be registered by our current methods of clinical evaluation. In addition, because the adult liver is relatively resistant to gross toxicity from Al, it may act as a sink for Al, preventing its accumulation in critical sites such as the brain and bone. For all of these reasons, cultured mouse hepatocytes (MH) were chosen as the primary cell system to perform our evaluation. In addition, we have established MH as a good model system that demonstrates Tf-associated Al uptake and toxicity (16). In this study, we sequentially examined the effect of 15 uremic HPLC fractions on the uptake of Al by cultured MH and identified several that enhanced Al uptake. We then tested known compounds.

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Correspondence to Dr. Kenneth Abreo, Department of Medicine, Louisiana State University Medical Center, Shreveport, LA 71130.
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extracted previously from these fractions for their effect on Al uptake in MH. Only p-cresol increased the Tf-associated uptake of Al in MH. p-Cresol also enhanced the uptake of Al in mouse neuroblastoma cells and Friend erythroleukemia cells, cell culture systems that also demonstrate Tf-associated Al uptake (17–19). The possible mechanisms by which these compounds enhanced hepatocyte Al uptake were also examined, pointing to either increased Tf receptor expression or cycling, or the limited egress of Tf-Al from the cell.

Materials and Methods
MH Cultures
MH, NCTC clone 1469 from American Type Culture Collection (Rockville, MD), were grown in medium NCTC 135 containing 10% horse serum and 160 µg/ml penicillin (regular media) at 37°C in a 5% CO₂ atmosphere to form a monolayer of cells in culture flasks (16). The MH clones used in these experiments are normal mouse liver cells that have a very low incidence of malignant transformation (20). The MH cells are similar to hepatocytes of the mouse strain of origin in glycogen content, beta glucuronidase activity, and arginase activity, but differ in catalase activity (21).

Preparation of Tf-Al and Uremic Fractions
Purified human Tf (98% pure; Sigma Chemical Co., St. Louis, MO) was saturated with Al as described by Trapp (22). Tf saturation with Al was 39.2 ± 3% (n = 4). Fifteen uremic extracts were obtained by HPLC (gradient from 100% formic acid, pH 4, to 100% methanol in 60 min), using a slight modification of the separation technique as described originally by Hsu et al. (23) and Vanholder et al. (24) from pooled hemodialysis ultrafiltrate (uremic fractions), and 15 extracts similarly eluted from ultrafiltrate of pooled normal plasma served as controls. Figure 1 shows the chromatogram of uremic fractions 1 to 15 obtained by HPLC.

Effect of Uremic Fractions on Al Uptake, Cell Growth, and Enzyme Leakage in MH
To evaluate whether uremic ultrafiltrate fractions enhanced the uptake of Al from Tf-Al in media, MH were plated at 10⁴ cells/ml in regular media with Tf-Al (500 µg/ml Tt, 133 µg/L elemental Al) and the addition of either a normal ultrafiltrate fraction (from 1 to 15), which served as a control, or a corresponding uremic ultrafiltrate fraction (from 1 to 15). Cell Al, cell counts, and media aspartate aminotransferase (AST) concentrations were measured at 24, 48, 72, and 96 h in culture. Cells were also grown in media containing uremic

Figure 1. Chromatogram showing uremic fractions 1 to 15 obtained by HPLC eluted at equal intervals over 60 min. Arrows (→) indicate fractions that enhance Al uptake in MH. Inset shows elution profile of pure compounds corresponding to the indicated peaks of the uremic fractions.
ultrafiltrate fractions 1 to 15 alone, and cell counts and media AST concentrations were measured at similar intervals to see whether these uremic fractions were toxic to MH. Samples of media and cells were collected by scraping at 24, 48, 72, and 96 h after initial plating. Cell numbers were determined, and cell Al (μg/L) was measured by atomic absorption spectrophotometry (16). AST concentrations in the media were measured by a quantitative, colorimetric determination (U/10^6 cells, Sigma Diagnostics, St. Louis, MO).

**Effect of Uremic Compounds on Al Uptake, Cell Growth, and Enzyme Leakage in MH**

Because uremic ultrafiltrate fractions 4 to 8, 12, 14, and 15 enhanced Al uptake in MH and because each fraction consists of many known and unknown compounds as shown in Figure 1, we next arbitrarily chose some compounds previously extracted from these fractions (Figure 1) and tested their effect on MH Al uptake. The effect of xanthine, tryptophan, hippuric acid, α-hydroxyhippuric acid, and p-cresol at concentrations found in uremic plasma were tested in this system. Although phenol is not present in significant amounts in the HPLC eluate and the compounds α- and m-cresol are not found in biological fluids, the effect of these compounds on Al uptake was also tested because of their structural similarity to p-cresol. In these experiments, MH were grown in regular media with the addition of Tf-Al alone, Tf-Al and a uremic compound, Al citrate alone, Al citrate and a uremic compound, or a uremic compound alone. Cell Al, cell counts, and AST concentrations in media were measured at 24, 48, 72, and 96 h in culture. The media concentration of elemental Al as Tf-Al or Al citrate was similar at 133 μg/L. Compounds added were as follows: xanthine (0.11, 0.75, 1.5, and 2.25 mg/dl), tryptophan (0.5 and 1.5 mg/dl), hippuric acid (10 and 30 mg/dl), α-hydroxyhippuric acid (8 and 24 mg/dl), p-, m-, and α-cresol (1.5, 2, 2.5, and 3 mg/dl), and phenol (0.05, 0.15, 0.20, and 0.30 mg/dl).

**Effect of p-Cresol on Al binding to Tf**

Increased Al uptake in MH could be the result of p-cresol-induced increased binding of Al to Tf. To test this hypothesis, purified human Tf (98% pure; Sigma Chemical Co.) was saturated with Al as described by Trapp (22). Al citrate (Pfaltz and Bauer, Waterbury, CT) was added to Tf in a ratio of 2 mol of elemental Al per mole of Tf in the presence of 0.04 M NaHCO₃ with or without the addition of p-cresol at concentrations of 1.5, 2, 2.5, and 3 mg/dl. The unbound Al was then removed by ultrafiltration, using an Amicon stirred ultrafiltration cell (W. R. Grace & Co., Danvers, MA). The Al concentration was measured in the solution before and after ultrafiltration, and Tf
Figure 3. The effect of compounds previously extracted from Al-enhancing uremic fractions on MH Al uptake at 96 h in culture. Significant increases in MH Al uptake were observed from media containing p-, o-, and m-cresol and phenol. Control media and media containing compounds contained similar concentrations of Tf-Al.

saturation with Al was calculated in the presence and absence of p-cresol.

Effect of p-Cresol on Tf-[125]Al Uptake by MH

Because p-cresol increased Tf-associated net Al uptake in MH, the rate of Tf-[125]Al uptake ([125] radioactivity in the cell being a measure of Tf uptake) was evaluated in MH in the presence and absence of p-cresol. MH were grown in the presence (at a concentration of 3 mg/dl) or absence of p-cresol in media for 96 h. At 24, 48, 72, and 96 h, samples of MH were harvested and incubated at 10⁷ cells/ml in PBG (phosphate-buffered saline with glucose) containing 1 μM Tf-[125]-Al in a water bath at 37°C. At 1-min intervals, 100-μl samples of cells were removed and the reaction was stopped by dilution with 0.5-ml of ice-cold PBG. Cells were washed two times with PBG, and cellular radioactivity was determined in an LKB gamma counter (Gaithersburg, MD). The cellular uptake of Tf-[125] was expressed as molecules per cell per minute.

Effect of p-Cresol on Al Uptake in Friend Erythroleukemia and Neuroblastoma Cells

To determine whether p-cresol also enhanced the uptake of Al in other cell lines in which Tf-Al uptake has been demonstrated by us (17–19), Al uptake studies similar to those described above were

Figure 4. Time–response curves showing that p-cresol at concentrations of 3 mg/dl increased MH Al uptake at 24, 48, 72, and 96 h from media containing Tf-Al (p-C + Tf-Al versus Tf-Al). p-Cresol did not increase Al uptake from Al citrate (AlCit versus p-C + Tf-Al). p-C, p-cresol; Tf-Al, transferrin-aluminum; AlCit, aluminum citrate.
Figure 5. Time–response curves showing that p-cresol at concentrations of 3 mg/dl inhibited MH growth at 24, 48, 72, and 96 h only in media containing Tf-Al (p-C + Tf-Al versus Tf-Al). p-Cresol alone (p-C versus NA) or the addition of p-cresol to Al citrate did not inhibit cell growth (p-C + AICt versus AICt). NA, no additions to media. Other abbreviations as in Figure 4.

Figure 6. Time–response curves showing that p-cresol at concentrations of 3 mg/dl, respectively, caused AST leakage into media at 24, 48, 72, and 96 h only in media containing Tf-Al (p-C + Tf-Al versus Tf-Al). p-Cresol alone (p-C versus NA) or the addition of p-cresol to Al citrate did not induce AST leakage from MH (p-C + AICt versus AICt).

Figure 7. Dose–response curves showing significantly increased hepatocyte Al uptake and inhibition of cell growth at p-cresol concentrations ≥2 mg/dl at constant media concentrations of Tf-Al containing 133 μg/L elemental Al.

Test, using Stat-View statistical software for Macintosh (Abacus Concepts, Inc.) (P < 0.05 was considered significant).

Results

Uremic Fractions Increase the Uptake and Toxicity of Al in MH

As shown in Figure 2, uremic fractions 4 to 8, 12, 14, and 15, when compared with control fractions, increased cellular Al uptake, increased AST release into media, and decreased cell growth at 96 h in culture. These uremic fractions were not toxic to MH in the absence of Tf-Al (data not shown). The
Figure 8. Rate of Tf-1^{25} uptake by MH from Tf-I^{25}-Al added to media. Cells were grown with or without p-cresol in the medium at a concentration of 3 mg/dl, and experiments were conducted with cells at 24, 48, 72, and 96 h after plating. Tf-I^{25} uptake was always significantly increased in cells grown with p-cresol in the medium (p-C + Tf-Al versus Tf-Al, P values shown). There was no significant difference in Tf-I^{25} uptake by MH grown in p-cresol (p-C + Tf-Al) at each time point tested.

Figure 9. Aluminum uptake by mouse hepatocytes (MH), Friend erythroleukemia cells, and neuroblastoma cells grown in media containing Tf-Al, and Tf-Al plus p-cresol at 96 h in culture. Concentration of Tf-Al (133 µg/L elemental Al) and p-cresol (3 mg/dl) was kept constant in all media.

remaining uremic fractions 1 to 3, 9 to 11, and 13 did not affect cell Tf-Al uptake or cause cell toxicity (not shown).

**p-Cresol Increases the Uptake and Toxicity of Al in MH**

Next, the compounds xanthine, tryptophan, hippuric acid, o-hydroxyhippuric acid, p-, o-, and m-cresol, and phenol, solutes known to elute from the Al uptake-enhancing uremic fractions 4 to 8, 12, 14, and 15 (Figure 1), were tested for their effect on Al uptake and toxicity in MH. Only the three cresol isomers and phenol significantly increased Al uptake in MH from Tf-Al, whereas Al uptake from Al citrate was not enhanced (Figure 3). Further studies on phenol and o- and m-cresol are not reported because phenol did not elute out of the Al-enhancing uremic fractions and o- and m-cresol are not detected in biological fluids. Figures 4 through 6 show that p-cresol, when added to Tf-Al, increased MH Al uptake and resulted in significantly greater cell toxicity when assessed by cell growth and AST leakage into media compared with MH grown in Tf-Al alone. There was no increase in MH Al uptake or cell toxicity when p-cresol was added to Al citrate (Figures 4 through 6). p-Cresol was not toxic when added to media without Tf-Al, as shown in Figures 5 and 6. Dose–response curves showed proportionately increased hepatocyte Al uptake (Figure 7), inhibition of cell growth (Figure 7), and increased media concentrations of AST (data not shown) at p-cresol concentrations >1.5 mg/dl. The cresol isomers o- and m-cresol caused increases in Al uptake and toxicity in MH, but to a significantly lesser extent than p-cresol (results not shown). Xanthine, tryptophan, hippuric acid, and o-hydroxyhippuric acid had no effect on Al uptake or toxicity on MH.

**p-Cresol Does Not Affect Al Binding to Tf**

Because the enhanced Tf-Al uptake in MH induced by p-cresol could have resulted from increased binding of Al to Tf, the effect of this compound on Tf saturation with Al was tested. There were no significant differences in the Tf saturation with Al in the absence (Tf saturation 33 ± 3.2%, n = 5) or presence of p-cresol at concentrations of 1.5 mg/dl (Tf saturation 36 ± 2.1%, n = 5), 2 mg/dl (Tf saturation 32 ±
3.9%, n = 5), 2.5 mg/dl (Tf saturation 31 ± 1.9%, n = 5), and 3 mg/dl (Tf saturation 31 ± 3.1%, n = 5).

**p-Cresol Increases the Uptake of Tf-\( {\text{Al}}^{125} \)-Al by MH**

To show definitively that p-cresol increased net cellular Al concentrations as a result of Tf uptake, the uptake of Tf-\( {\text{Al}}^{125} \)-Al was assessed in MH. The mean net uptake of Tf-\( {\text{Al}}^{125} \) was significantly increased in the presence of p-cresol in the medium at 24, 48, 72, and 96 h in culture (Figure 8).

**p-Cresol Increases Al Uptake in Friend Erythroleukemia and Neuroblastoma Cells**

Because Tf receptors are found on numerous cell types, the effect of p-cresol was tested in other cell lines in which Tf-AI has been shown (17–19). p-Cresol increased Tf-associated uptake in Friend erythroleukemia cells and neuroblastoma cells (Figure 9). The percentage increase in Al uptake was similar for all three cell lines. The magnitude of net cellular Al accumulation was proportional to the number of receptors expressed by each cell line in that Friend erythroleukemia cells accumulated the most Al and neuroblastoma cells accumulated the least (Figure 9).

**Discussion**

To our knowledge, there are no studies in the literature in which the effect of specific uremic compounds on cellular or whole organ uptake of Al has been evaluated. Of the 15 uremic extracts tested, fractions 4 to 8, 12, 14, and 15 enhanced MH Al uptake (Figure 2A). Because these extracts were obtained by HPLC of pooled hemodialysis ultrafiltrate, each contained a large number of known and unknown compounds. Known compounds previously extracted from these fractions were then tested at the concentrations found in the serum of ESRD patients for their effect on Al uptake in MH (Figure 3). Of the compounds tested, only p-, m-, and o-cresol (at concentrations of 1.5 to 3 mg/dl) and phenol (at concentrations of 0.05 to 0.30 mg/dl) enhanced Tf-associated Al uptake, whereas xanthine, tryptophan, hippuric acid, and o-hydroxyhippuric acid had no effect on Al uptake (Figure 3). Even though insufficient amounts of phenol elute from HPLC extracts of hemodialysis ultrafiltrate due to its protein binding, this compound was tested because of its structural similarity to cresol. o- and m-Cresol also were tested because they are isomers of cresol even though they are not found in biological fluids. Detailed studies on phenol and o- and m-cresol are not reported because they may not be clinically relevant.

p-Cresol at concentrations found in dialysis patients was toxic to MH in the presence of Tf-Al as assessed by cell growth and AST leakage into media (Figures 5 and 6). This compound was not toxic to MH per se, because no cell toxicity was seen in the absence of Tf-Al (Figures 5 and 6). We have shown previously that sufficient AI is taken up by MH from Tf-Al alone, even in the absence of p-cresol, to cause cell toxicity (16). The addition of p-cresol to Tf-Al significantly increased net Al uptake by MH and thereby exaggerated the Al toxicity (Figures 4, 5, and 6). Dose–response curves also showed that Al uptake in MH (Figure 7) and cell toxicity (Figure 7) were proportional to the concentration of p-cresol in the medium. These findings offer additional evidence that these uremic solutes enhance Al uptake in MH and that cellular toxicity results from Al rather than a direct effect of this compound.

The serum concentration of p-cresol is persistently elevated in ESRD patients for two reasons: (1) because it is continuously synthesized from tyrosine in the gut by anaerobic bacteria such as Clostridium and Bacteroides (25); and (2) the renal clearance of p-cresol is impaired in ESRD, resulting in its accumulation in blood (25, 26). The poor dialyssance of this low molecular weight compound (108 D) suggests that it is probably highly protein bound (25). As reported by Niwa, the posthemodialysis reduction rate is only 37% for p-cresol (25). Our preliminary results also show a 33% reduction in p-cresol with hemodialysis (27). These persistently elevated levels of p-cresol would potentiate the cellular deposition of Al whenever ESRD patients are exposed to this metal.

Our studies show that p-cresol only enhances Tf-associated Al uptake in MH. Because Al uptake from Al citrate in the medium was not increased (Figure 4) by this lipophilic compound, it is unlikely that it rendered the cell membrane more permeable by altering its structure. Al citrate was selected because it has been suggested as the most probable small molecular ligand for the nonprotein-bound fraction of serum Al (28). The precise mechanism(s) for enhanced Tf-Al uptake by MH is not completely elucidated. One potential mechanism could be p-cresol-induced increased saturation of the carrier protein Tf with Al. However, our studies showed that p-cresol did not increase the binding of Al to Tf. Increased uptake of Tf-\( {\text{Al}}^{125} \) was observed when Tf-\( {\text{Al}}^{125} \) was added to media together with this compound (Figure 8). Increased Tf receptor expression, a faster rate of Tf receptor cycling, or limited egress of Tf-Al from the cell could result in the increased Al accumulation in MH.

Cellular receptors to Tf are ubiquitous and vary in number with cell type, and Tf-associated Al uptake has been demonstrated in many cultured cells (29). Therefore, the effect of p-cresol on Tf-Al uptake was tested in Friend erythroleukemia and neuroblastoma cells in which we have previously shown Tf-Al uptake (17–19). Uptake of Al was augmented by p-cresol in both cell types, suggesting that Al would accumulate in all cells expressing Tf receptors. The Al taken up by the cell lines tested correlated with the number of receptors expressed by each. Friend erythroleukemia cells accumulated the highest and neuroblastoma cells accumulated the lowest concentration of cell Al (Figure 9). The metabolic changes noted in MH may therefore be relevant to all cell systems in which Al is absorbed in excess.

Although a number of substances not normally found in normal individuals have been extracted from the serum of ESRD patients (30), no in vitro or in vivo studies have been done with these putative uremic toxins to ascribe a particular pathophysiologic role in cellular Al uptake. The studies described here show for the first time that several uremic fractions and the compound p-cresol in particular enhance the cellular uptake of Al in MH. p-Cresol also plays a role in uremic coma and bleeding tendencies (26) and in the depressed
immune function of ESRD patients by inhibiting phagocyte function (31). It remains to be shown whether these compounds also enhance the gastrointestinal absorption and organ deposition of Al in ESRD patients, thereby providing a partial explanation for the vulnerability of certain subsets of ESRD patients to Al overload.

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