Binding of Al by Protein in Plasma of Patients on Maintenance Hemodialysis

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Gel filtration of plasma from hemodialysis patients, with use of reagents and apparatus with carefully minimized background Al concentrations, reproducibly showed a single peak for Al, corresponding exactly to the elution position of transferrin. The Al/transferrin molar ratio in adjacent fractions was constant (mean 0.126, SE 0.006) in replicate experiments. In contrast, the association of Al with albumin varied. Using both equilibrium dialysis and gel-filtration techniques, in the presence and absence of calcium or phosphate, we could demonstrate no significant binding of Al by human albumin at Al concentrations of 1 to 12 μmol/L. We saw no Al peak in pooled, concentrated, low-molecular-mass fractions of plasma gel-filtered on Sephadex G-50. Evidently, transferrin is the sole Al-binding protein in plasma of hemodialysis patients.

Additional Keyphrases: gel filtration · transferrin as Al carrier in plasma · trace elements · toxicology

Aluminum in plasma usually increases during hemodialysis, apparently against a concentration gradient (1–3), which is consistent with the metal being bound by protein. Reports (4, 5) that 60 to 80% of the Al in plasma is associated with the large molecules support this view. It is now clear Al interacts with transferrin in plasma (6–8), but it has also been suggested (7, 9) that there is substantial binding to albumin. The high concentration of albumin in plasma as compared with that of transferrin makes it important to determine the relative roles of these (and any other) proteins as carriers of the metal.

Here, we have used gel-filtration techniques to obtain strong evidence that transferrin is the sole protein carrier of Al in the plasma of maintenance-dialysis patients.

Methods

Al in fractionated plasma: Plasma sampled after dialysis from three maintenance-hemodialysis patients was pooled, diluted 10-fold with buffer, then concentrated to two-thirds its original volume by use of Amicon UM2 membranes. The pH 7.4 buffer, prepared from "Suprapure" chemicals (Merck, Darmstadt, F.R.G.), contained, per liter, 25 mmol of Tris HCl, 100 mmol of NaCl, and 10 mmol of NaHCO₃ in doubly-distilled water. Its Al concentration was below our detection limit of 0.2 μmol/L. We applied 1.5-mL samples of the concentrated plasma to a 100 × 1.6 cm column of Sephacryl S-300, carrying out the gel filtration with apparatus shielded from dust. We monitored the protein content of the eluent by its absorbance at 280 nm. Five successive samples of the pooled plasma were so tested, and between each procedure 0.5 mL of a 10 mmol/L solution of EDTA (pH 5.5) was passed through the column to remove any residual Al. Five identical sets of 1.7-mL fractions were thus collected and their Al content was estimated by electrothermal atomic absorption spectroscopy. For protein assay we used electrophoresis on agarose gel. Transferrin and albumin were measured by immunodiffusion (Partigen system; Behring, Marburg, F.R.G.).

Elution of human transferrin and Al: Human transferrin (Sigma Chemical Co., St. Louis, MO) was dissolved in the buffer to give a 10 mg/mL solution and 100 μL of a 1.0 mmol/L stock solution of AlK(SO₄)₂ was added to give a final Al concentration of 100 μmol/L. After a 30-min equilibration, the solution was diluted 20-fold with the buffer, then concentrated to its original volume by use of an Amicon UM2 membrane, to minimize non-bound Al. The transferrin and Al concentrations were then estimated. We applied 0.1 mL of this solution to the S-300 column and measured transferrin and Al in the effluent as before.

Al binding with low-molecular-mass fractions: Pooled plasma samples from three hemodialysis patients were applied in 5-mL portions to a 50 × 2.5 cm column of Sephadex G-50. Effluent corresponding to the void volume was discarded. Those fractions of effluent that contained material of relative molecular mass (M_r) approximately 2000 to 25 000 were pooled, concentrated 20-fold, and 1-mL portions were then successively applied to a 100 × 1.6 cm column of Sephadex G-50 and the fractions analyzed for Al.

Affinity of S-300 and G-50 gels for Al: We treated four samples of plasma (two normal, two uremic) with the AlK(SO₄)₂ solution, 5 μL per milliliter of plasma, and estimated the final Al concentration. We mixed 0.5-mL portions of the treated plasma with 2.5 mL of a slurry of S-300 gel, centrifuged the mixture (2000 × g, 5 min), removed the supernate, and analyzed it for Al. The gel was then mixed with 0.5 mL of a 10 mmol/L solution of EDTA, pH 5.5, centrifuged, and the Al concentration in the supernate estimated. We repeated the experiment, using G-50, and also with buffer containing 10 μmol of Al per liter in place of the plasma.

Interaction of Al with albumin: We prepared 4 g/L stock solution of crystallized human albumin (Sigma) in the buffer and dialyzed 1-mL portions against the buffer, which in this case was modified to contain Al in concentrations ranging from 0 to 12 μmol/L. For this, we used a multi-cell dialyzer (MSE, Zurich, Switzerland) with Spectrapor 2 membranes (Spectrapor, Los Angeles, CA), dialysis being for 3 h in a water bath set at 37°C. Similar experiments were carried out with CaCl₂ added to the buffer to give a final calcium concentration of 0.2 mmol/L and, separately, with neutral phosphate buffer added to give a final phosphate concentration of 0.5 mmol/L in the buffer.

After the dialysis, we collected the fluids on each side of the membrane and analyzed them for Al. We also did this experiment in triplicate with albumin-free buffer, to see whether Al had equilibrated across the membrane.

We tested the possibility of adsorption of Al on dialysis membranes by preparing, in duplicate, a series of 5-mL solutions of the buffer containing five different Al concentrations ranging from 1 to 12 μmol/L, in identical containers. One of each was shaken overnight with a Spectrapor membrane and the final Al concentration was then compared with the original.

The interaction between Al and albumin was also exam-
ined by elution from a Sephacryl S-300 column. The 30 x 1.6 cm column was equilibrated with the buffer modified to contain Al as AlK(SO₄)₂, and 20 mg of albumin was applied. The effluent was collected and the albumin concentration in the fractions measured by absorbance at 630 nm, after binding of bromcresol green (10). We carried out the experiment at three different Al concentrations (1, 3, and 12 μmol/L), in the absence and presence of 0.1 mmol of CaCl₂ per liter and, separately, of 0.5 mmol of phosphate per liter. We analyzed fractions preceding, including, and following the albumin peak for Al. In those experiments performed without phosphate, the concentration of phosphate in the solutions was adjusted to 0.5 mmol/L before analysis for Al. All membranes were pre-cleaned with three washes of buffer. The glassware was thrice washed in 100 mmol/L HNO₃ and then in distilled water, but plastic containers were used where possible. All experiments were carried out at 25°C unless otherwise stated.

The protocol used for estimating Al in biological samples with the Varian-Techtron CRA 90 graphite-furnace atomic absorption spectrophotometer (Varian, Kilkenny, South Australia): sample size, 10 μL; drying, 120 °C for 5 s; ashing, 1500 °C for 20 s; atomization, 2500 °C for 2 s; ramp rate to atomization, 400 °C/s.

**Results**

The concentrated sample of pooled plasma had a final Al concentration of 14 μmol/L and yielded the three usual major peaks for protein on gel filtration. After five successive identical procedures we found high Al concentrations only in the third peak, where the concentration rose progressively to a maximum of 0.87 (SE 0.09) μmol/L before declining rapidly in succeeding fractions to the background value of 0.25 (SE 0.04) μmol/L. We observed a variable increase in Al concentration up to 1.20 μmol/L in the volume corresponding to the salts, but no clearly reproducible peak. Figure 1 shows the Al concentration in each fraction in relation to the absorbance tracing. The proteins in those effluent fractions containing Al were predominantly albumin and transferrin, with small amounts of haptoglobin. The albumin and transferrin could be readily distinguished by immunodiffusion assay, allowing calculation of the mean ratios of Al to transferrin, and of Al to albumin, for each fraction. Corresponding fractions showed a highly reproducible association between Al and transferrin, with an almost constant mean value of 0.12 for the molar ratio (Table 1); in contrast, there was no such consistent relationship between Al and albumin.

After gel filtration of the transferrin–Al complex, which had been prepared in vitro, analytical recovery of transferrin was 98%, but the Al in the same fractions was only 33% of the initial load, with the Al–transferrin molar ratio declining from 1.04 to 0.34. The remainder of the Al could be accounted for in the salts volume.

When we eluted the samples prepared to contain only the low-M₇ components of plasma on G-50 in repetitive experiments, a variable peak for Al was occasionally observed in the void volume but no Al was detected in the subsequent fractions. Sephacryl S-300 and Sephadex G-50 retained no Al after exposure to either plasma or buffer, and subsequently no Al could be extracted from them with EDTA.

Crystallized human albumin (Sigma) was found to be Al-free and to contain only 1 g of transferrin per kilogram. In the control dialysis experiment, the Al concentrations on the side of the membrane that was initially free of Al had increased to 85 to 95% of the final Al concentration in the dialysate by the end of the procedure. The Spectrophor membranes adsorbed about 40% of the Al in the buffered solutions tested. However, it could be shown that when albumin was dialyzed against final Al concentrations of 1 to 4 μmol/L, the resulting Al/albumin molar ratio was 0.030 (SE 0.007) after allowance for the background concentration (n = 7), if no calcium or phosphate was present. In the presence of either calcium (n = 6) or phosphate (n = 6) there was no association between Al and albumin (molar ratio 0.005 (SE 0.005)). Albumin filtered through an S-300 column that had been equilibrated with buffer containing Al showed no tendency to bind the metal at any of the concentrations tested, whether or not calcium or phosphate were present.

The elution profile was very similar for each run, and an example is shown (Figure 2). The Al concentration in the effluent was relatively constant despite a clear albumin peak. In repeated analyses of a sample from the gel filtration of pooled plasma on S-300, the CV for Al estimation was 6% between batches, based on 15 observations. The coefficient of correlation for standard addition of Al in plasma (range 0–16 μmol/L) was 0.997 with gradient 0.887.

**Discussion**

These findings confirm the previous reports by ourselves (6, 8) and others (7) that transferrin carries Al in the plasma of patients after maintenance dialysis. Our use of replicate gel filtration to increase the precision of our observations shows there is a single peak for the metal, corresponding exactly to eluted transferrin as identified by immunodiffusion, and this association is emphasized by the strict stoichiometric relationship between the metal and the protein in the fractions corresponding to this peak (Table 1). The experiment designed to elute an Al–transferrin complex on S-300 showed that, during passage down the column, there was substantial dissociation of the complex, because only a third of it was accounted for in combination with the
protein. However, we saw no evidence of an affinity between Al and the gel, implying that instead there was a degradation of some binding sites during the procedure.

It has been suggested (7, 11) that Al may be specifically carried on albumin, and even if the complexing were relatively weak, the high molar concentration of albumin in plasma would make this protein a potentially important carrier. However, we employed our method of repetitive gel filtration to examine no substantive association between Al and albumin, because the Al/albumin ratio was variable and the values for the means were exceeded by the corresponding standard errors. Nonetheless, to see if the Al could have dissociated from the albumin during the gel filtration with Al-free buffer, we examined the potential for association between the protein and Al by equilibration with buffer containing Al, using both dialysis and gel filtration. Because of the possibility that, in the clinical setting, calcium in plasma might affect binding of Al to albumin, a set of experiments was conducted with calcium and albumin present in the physiological molar relationships. Conversely, the relative excess of phosphate in plasma might complex free Al in vivo, so we did a further group of experiments examining the Al–albumin interaction, using concentrations of Al and phosphate approximating those that exist in clinical circumstances. In the dialysis experiments, despite difficulties with the procedure, interaction between Al and human albumin was apparently negligible and absolutely no evidence of complexing was observed when either calcium or phosphate was present. The absence of binding of Al by albumin is further supported by our results for equilibration of albumin with different Al concentrations by use of gel filtration. Once again, there was no detectable binding of the metal by albumin whether calcium or phosphate were present or absent (Figure 2).

It has been suggested (9) that there is at least one low-M₈ Al-binding protein, a suggestion arising from data from a single gel filtration procedure. However, in agreement with another report (7), we found no Al peak in the G-50 effluent containing low-M₈ components. Since we were unable to demonstrate any affinity between G-50 and Al, which might have obscured weak complexing, we think that there is also no Al–protein association.

How is Al bound by the plasma proteins? This question is of obvious interest because it may influence our approach to the prevention of Al intoxication in renal-failure patients, and may conceivably shed light on other aspects of Al metabolism. Our findings strongly suggest that transferrin is the sole protein carrier of Al in plasma; our recent report (12) showing the high-affinity binding at two sites on that molecule also casts transferrin in the role of specific carrier for this metal. Thus, if binding sites are available, transferrin will act as a "sink" for most Al entering the plasma, either during dialysis (1) or after gastrointestinal absorption, greatly inhibiting its removal by hemodialysis (13) unless the metal is bound to a low-M₈, chelating agent such as desferrioxamine (14). By contrast, some loss of bound Al would be expected on peritoneal dialysis (15). A proportion of the Al in plasma is ultrafiltrable (4, 5), and this moiety might appear in the urine. Although we have no data as to the fate of the transferrin-bound Al, it is reasonable to suppose that, after degradation or other conformational change (16), there would be a slow exchange with tissue sites, where there may be fixed metal-binding proteins, or with hydroxyapatite in the bone. It also might be delivered specifically to sites where it could interfere with metabolism of iron or other trace elements.

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References