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Ferritin: Isolation of aluminum–ferritin complex from brain

(ferritin/aluminum toxicity)

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ABSTRACT **Ferritin was isolated from the livers and brains of two groups of rats, one of which was fed aluminum chloride (100 μM) for 1 year in the drinking water. Brain tissue contained about one-third of the amount of ferritin found in the liver. While brain ferritin from normal rats contained 42.1 ± 14.3 mol of aluminum, that from the aluminum-fed group contained 115.4 ± 48.3 mol of aluminum per mol of ferritin. Liver ferritin from both groups contained similar amounts of both aluminum and iron, and the amounts were less than that found associated with brain ferritin. Ferritin isolated from the brains of patients who died of Alzheimer disease contained more aluminum and more iron than that from age-matched controls. Human brain ferritin is composed of two types of subunits—about 70% heavy chain (M_r, 22,000) and 30% light chain (M_r, 19,500). The isoelectric focusing pattern of human brain ferritin was considerably different from that of human liver. Only 5 of the 20 brain ferritin bands migrated similarly to the acidic isoferritins from the liver, and the major component of brain ferritin, representing 30% of the total ferritin, had a pI of 8.0.**

Ferritin is a ubiquitous iron (Fe)-storage protein with a M_r of ≈480,000. It is composed of two types of subunits—heavy (H; M_r, 21,000) and light (L; M_r, 19,000) chains (1). Ferritins from heart and liver contain predominantly H or L chains, respectively. Synthesis of ferritin is enhanced by Fe^{III} (2). A fully saturated ferritin contains up to 4500 mol of Fe sequestered as ferrichydroxyphosphate in its protein shell (1). Since 1982 the data obtained by us and by other laboratories suggest that ferritin is a multifunctional molecule, and as such (*i*) it detoxifies, stores, and transports Fe; (*ii*) it binds other metal ions both *in vivo* and *in vitro* (3, 4) and functions as a zinc (Zn) detoxicant and a Zn ion donor (5); (*iii*) in the presence of a reductant, it releases Fe^{II}, which can facilitate the generation of free radicals (6, 7); and (*iv*) it functions as a dephosphorylating agent and therefore may affect several metabolic processes (6, 8). We found that holoferritin can bind unusually large quantities of beryllium (Be) and that rats pretreated with Fe injections resulting in elevated levels of ferritin survived otherwise toxic doses of intravenous Be (4). An expanded role for ferritin in metal toxicity was thus suggested (4, 8, 9).

Although the chemical properties of Be and aluminum (Al) are similar (10), Be is extremely toxic (11), but Al, one of the most abundant elements in the earth's crust and in the environment, until recently has been considered harmless. The reported increased Al levels in the brains of patients with dialysis dementia (12) and Alzheimer disease (AD) (13) have led to the suggestion that Al may be an etiological agent for dementia. Other current theories to explain the pathogenesis of AD include decreased cholinergic innervation, defective protein synthesis, and a slow infectious process (14). None are unequivocally proven or ruled out.

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Humans ingest about 30 mg of Al daily, mostly as a food contaminant, but only about 10–20 μg is actually absorbed (15). A substantial amount of the absorbed Al is deposited in the bones and brain (15). Thus, under normal physiological conditions, the accumulation of Al results from chronic low-level exposure. The isolation of ferritin complexed with Be, Zn, copper, or cadmium from the livers of animals injected interperitoneally with the corresponding metal salts (8) and the similarities between the inorganic properties of Be and Al suggested that Al–ferritin complexes might be obtained from the brains of animals that were fed even low levels of Al.

Therefore, we initiated a series of experiments to study the long-term effects of low levels of dietary Al on the ferritin in the brain and liver. In this report we show that (*i*) normally, a significant amount of Al in the brain is complexed with ferritin; (*ii*) the ferritin isolated from the brains of Al-fed animals has more Al bound to it than ferritin isolated from control animals; (*iii*) the ferritin isolated from the brains of AD patients has more Al bound to it than ferritin isolated from the age-matched patients without AD; and (*iv*) compared to liver ferritin, the isoelectrophoretic pattern of brain ferritin displays greater microheterogeneity and contains less iron. Some of these feelings have been presented at recent meetings (16–18).

MATERIALS AND METHODS

The water used was passed through two ion exchangers and had a resistance of more than 1.5 MΩ. Ultrapure HNO_3 was obtained from Baker. Metal standards used for atomic absorption spectrophotometry were obtained from Spex Industries (Metuchen, NJ). All gel components were obtained from Pharmacia. All other chemicals were of analytical grade. All glassware and plasticware were rinsed with 2% HNO_3 and deionized water before use.

To determine realistic levels of Al to be used in the animal experiments, we measured the Al content of six common beverages in bottles and cans randomly collected from local grocery stores. The pH of each sample was determined. Aliquots (1 ml) of each were degassed under a vacuum, adjusted to 2% HNO_3, and analyzed for Al. The amount of Al was determined from solutions adjusted to 2% HNO_3 with an IL-550 atomic absorption spectrometer equipped with a graphite furnace.

Twenty-two male 10- to 12-week-old Sprague–Dawley rats obtained from Charles River Breeding Laboratories were divided into two groups and fed *ad lib* Purina Rodent Laboratory Chow 5001 and tap water. The solid food was prepared for Al analysis by digestion in 50% HNO_3 for 12 hr at 70°C, and the digest subsequently was diluted to 2% HNO_3. The tap water was prepared for analysis by adding HNO_3 to 2%. The Al content of the chow and water was 8.3 mg/g dry weight and 2.7 μM, respectively. The drinking water of the

Abbreviations: AD, Alzheimer disease; L, light; H, heavy; IEF, isoelectric focusing.

experimental group was supplemented with 100 μM $AlCl_3$. This was about 3 times the Al concentration measured in the commercial beverages. After one year the animals were tested in a standard T-maze for learning and recall (19) and sacrificed; their brains and livers were dissected, rinsed with ice-cold saline, and stored until used at −80°C in 0.1 M Tris·HCl (pH 7.4).

Ferritin was isolated from the homogenates of two brains (2 g per brain) and from individual livers as described (3) up to the point of precipitation with $(NH_4)_2SO_4$. The 40–50% saturated $(NH_4)_2SO_4$ precipitate was dissolved in a minimum volume of 5 mM Na_2HPO_4 buffer (pH 7.0) and applied to a 1.8 × 75 cm Sephacryl S-300 column equilibrated with the same buffer; 3-ml fractions were collected with UV monitoring and assayed for Fe. The Fe-containing fractions were pooled, concentrated with an Amicon concentrator fitted with a PM-30 filter, and stored at 5°C with 0.05% sodium azide. The isolated protein was shown to be ferritin by immunoprecipitation against polyclonal rabbit antibodies against the H chain of human liver ferritin.

The brains from four elderly patients, two of which displayed the histological features of AD as determined by light microscopy, were obtained through the Cole Neuroscience Foundation. Their age, sex, and cause of death were as follows: (*i*) 78 years, female, small bowel volvulus with jejunal infarction and AD, (*ii*) 80 years, female, respiratory failure due to bilateral acute necrotizing bronchopneumonia and AD, (*iii*) 78 years, female, septic shock secondary to urinary tract infection, and (*iv*) 55 years, male, myocardial infarction. Upon autopsy, the brains were removed, put in plastic bags, and frozen at −80°C. The whole brains were slightly thawed, cut into pieces, and homogenized in a blender for 1 min in 0.1 M Tris·HCl (pH 7.4). Ferritin was isolated by the same procedure outlined for rat brains.

Native PAGE was performed by using a 5% gel/barbituric acid/Tris system (20), and $NaDodSO_4$/PAGE was performed by using a 10–20% gradient gel with the ammediol system of Wychoff as cited in ref. 21. The amount of protein isolated was determined by the method of Lowry with the appropriate correction factors (3).

Isoelectric focusing (IEF) was carried out at 10°C on a Pharmacia FBE-3000 flat-bed system with a Pharmacia Ephortec 3000-V power supply. Initial attempts at IEF of brain ferritin using the standard techniques (Pharmacia handbook on isoelectric focusing), such as 5% acrylamide or 1% agarose gels under high-voltage and constant-power conditions, gave poor results. Electrophoresis for extended periods of time at low voltage, as described below, was essential to minimize smearing and obtain highly resolved brain ferritin bands. Therefore, the most satisfactory and reproducible procedure that resulted after several trials is described here in detail. Gels, 0.75 mm thick, were cast between glass plates, the inner face of one having been coated with dimethylsilane. The gel composition was 3% acrylamide/0.09% methylenebisacrylamide/6.3% 3–10 pH ampholyte/25% glycerol/0.04% *N,N,N′,N′*-tetramethylethylenediamine/0.02% ammonium persulfate. Electrode strips (2.5 × 5.0 mm) were placed 14 cm apart, and 0.1 M NaOH and 1 M acetic acid were used as cathodic and anodic electrolytes, respectively. The gel was prefocused for 3 hr at 10°C, during which time the voltage was gradually increased from 30 to 200 V. Filter paper strips were soaked (0.5 × 1.0 cm) in the appropriate ferritin solution and applied with forceps in duplicate to the gel 2 cm from the cathode. The gel was run at 100 V for 5 hr, after which the filter paper strips were removed and the gel was allowed to run overnight (14 hr) at 10°C. The next day the voltage was gradually increased over a 5-hr period to 2000 V, after which electrophoresis was stopped. Focusing time was determined as the time required for a sample placed at both the anode and cathode to migrate to the same pI region. The gel was placed in a pan over a thin layer of dry ice, 5-mm-wide slices from one lane were placed individually into 1.5-ml centrifuge tubes, and 0.5 ml of H_2O was added to each tube and left overnight. The next day the pH of each sample was determined, and concentrated HNO_3 was added subsequently to bring each tube to 2% HNO_3. The tubes again were left overnight, and the iron and aluminum content of each sample was determined. The rest of the gel was fixed in 5% thiosalicylic acid/10% trichloroacetic acid for 1 hr, soaked in methanol/acetic acid/water, 3:1:6 (vol/vol), stained with Coomassie blue, destained, dried between sheets of dialysis cellophane, and scanned on a Hoefer densitometer.

Equilibrium dialysis was performed with 1 mg of ferritin from human brain, human liver, and horse spleen in 1 ml of 5 mM Hepes buffer (pH 6.5). These samples were dialyzed at room temperature three times for 12 hr each against 2 liters of the same buffer containing 20 μM $AlCl_3$. The unbound Al was removed by further dialysis against the same buffer without Al. A dialysis bag containing aluminum-free buffer was processed simultaneously to serve as a blank. The contents of the bags were centrifuged, and the supernatant was then analyzed for protein, Fe, and Al by atomic absorption and for phosphate by colorimetry (22). Ferritin will precipitate at concentrations above 0.2 mM $AlCl_3$ (data not shown), but no precipitation was observed at 20 μM $AlCl_3$.

Statistical analysis was performed using a Hewlett–Packard HP-41 T-statistics program.

RESULTS AND DISCUSSION

Selecting a "low"-exposure level of Al is difficult because of its ubiquitous presence in the environment. Common sources include dust, smoke, medications, and water. Al levels in water fluctuate widely depending on a number of seasonal and climatic factors (23), as illustrated by the variation in Al content of Tennessee's surface waters (the 1984 U.S. Geological Survey Water Data Report states that Al concentrations in Tennessee surface waters vary between 10 and 230

Table 1. Effect of long-term Al feeding on rat ferritins

Group	Tissue	*n*	Analysis*		
			Ferritin, μg/g (wet weight) of brain	Element, mol/mol of ferritin	
				Fe	Al
Control	Liver	5	152.6 ± 31.2	1257.7 ± 276.4	4.6 ± 1.8
	Brain	5	49.1 ± 9.5	1036.8 ± 350.3	42.1 ± 14.3
Al fed	Liver	5	241.6 ± 54.3†	1262.5 ± 287.1	4.3 ± 2.3
	Brain	6	53.7 ± 8.8	1505.0 ± 326.5‡	115.4 ± 48.3§

*Values are means ± SD.
†Significantly greater than the control ($P < 0.02$).
‡Significantly greater than the control ($P < 0.05$).
§Significantly greater than the control ($P < 0.01$).

Table 2. Al and Fe in the ferritin from normal and AD human brain

Source	n	Brain sample, g	Analysis*		
			Ferritin, μg/g (wet weight) of brain	Element, mol/mol of ferritin	
				Al	Fe
Normal	2	1154.5 ± 47.0	38.1 ± 13.6	3.4 ± 0.6	435.6 ± 38.3
AD patient	2	1082.0 ± 21.5	60.4 ± 8.3	18.9 ± 0.4†	579.5 ± 3.7‡

*Values are means ± SD.
†Significantly greater than normal ($P < 0.01$).
‡Significantly greater than normal ($P < 0.05$).

ppb; ref. 24). As a first approximation to an experimental dose, we determined the Al content of some common beverages sold in bottles and cans. Considering the multiple sources of chronic Al exposure, an arbitrary experimental dose of 100 μM was decided upon.

After 1 year the control and Al-fed rats were tested in a T-maze for learning and recall. Although the Al-fed rats tended to require more time and made more errors to reach the food source, the results were only on the borderline of statistical significance (data not shown). This is not surprising in view of the well-known resistance of rats to Al-induced behavioral changes (25). The average weight of the control rats was 620.6 ± 24.4 g and that of the Al-fed group was 598.6 ± 25.5 g. The average weight of the control and Al-fed livers was 21.4 ± 3.4 g and 21.6 ± 2.8 g, respectively. The average weight of the brains of the control and Al-fed rats was 1.91 ± 0.14 g and 1.99 ± 0.11 g, respectively. There were no significant differences between the two groups for body, liver, and brain weights.

Consistent with the earlier observations (26), the brains of the Al-fed group contained more Al (data not shown), but, more importantly, some of this was sequestered by ferritin (Table 1). Brain ferritin from the control group contained 42.1 ± 14.3 mol of Al per mol of ferritin, but that from the Al-fed group contained 2.7 times more ($P < 0.01$). The higher ferritin content of the brains from the Al-fed rats was statistically insignificant.

The liver ferritin from both the control and Al-fed rats contained far less Al than the corresponding brain ferritin from each group. Additionally, 1.4 times more Fe was bound to the ferritin from the brains of the Al-fed animals ($P < 0.05$). In contrast with brain ferritin, the total liver ferritin from the Al-fed animals was 1.5 times more than that from the controls ($P < 0.02$). Therefore, in rats, increased Al intake produced an increase in Al and Fe bound to brain ferritin, and more liver ferritin appears to have been synthesized.

In view of the suspected role of Al in the etiology of Alzheimer disease and encouraged by the differences in Al content of the ferritin in rat brain and liver, we isolated ferritin from the brains of two AD patients and two nondemented patients and quantified the Al and Fe content of ferritin (Table 2). Human brain contained a substantial amount of ferritin, almost 30% of that found in human liver, and the ferritin from AD brains contained 5.6 times more Al than the controls ($P < 0.01$). In addition, the ferritin from AD brains contained 33% more Fe than the ferritin from patients without AD ($P < 0.05$). Although the total amount of isolatable ferritin from both rat and human brain averaged approximately 50 μg/g of brain tissue, the greater mass of human brain tissue yielded much more ferritin for further

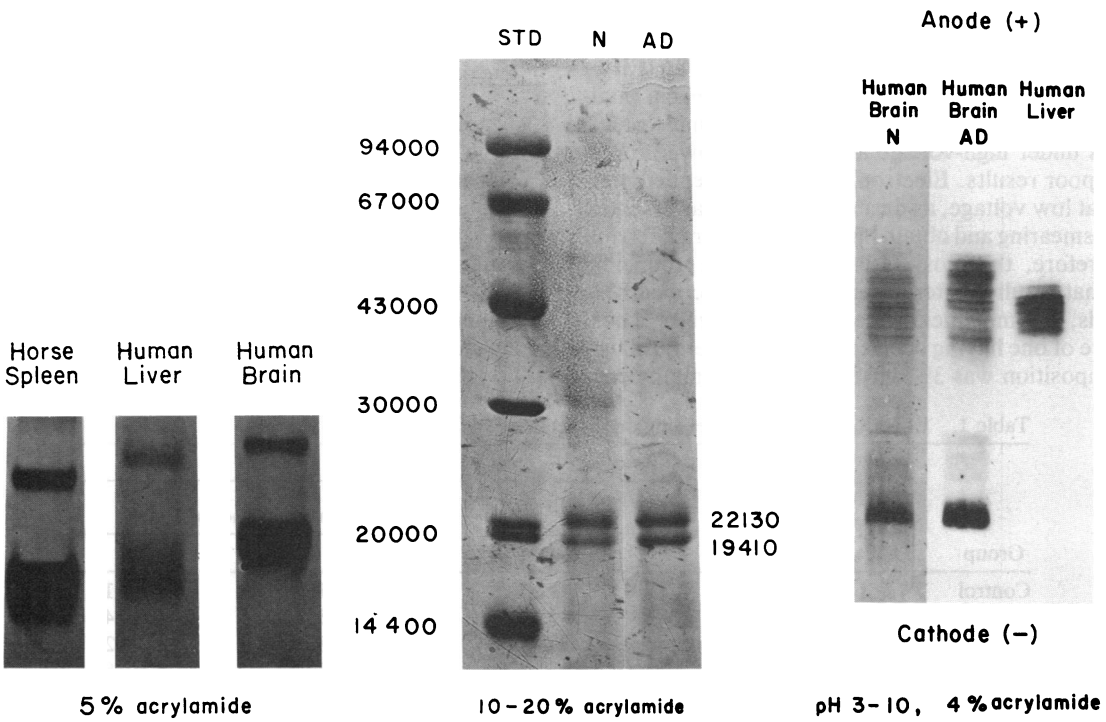

FIG. 1. Electrophoresis of human ferritin. (*Left*) Native PAGE. (*Center*) In gradient $NaDodSO_4$/PAGE. Lanes: STD, molecular weight standards; N, ferritin from normal human brain; AD, ferritin from AD brain. (*Right*) IEF. N, normal. For conditions, see the text.

characterization. The molecular weight of human brain ferritin was determined to be about 500,000 by gel filtration (data not shown). Upon electrophoresis, under nondenaturing conditions, ferritin from human brain, liver, and horse spleen showed different mobilities (Fig. 1 *Left*). All three preparations also contained a slower moving iron-containing minor band representing a dimer of ferritin. In $NaDodSO_4$, human brain ferritin dissociated into H and L subunits of M_r 22,130 and M_r 19,400, respectively (Fig. 1 *Center*).

Densitometric scanning of the gels showed that the relative proportion of the H and L chains was approximately 70% and 30%, respectively, for both normal and AD ferritin (data not shown).

Ferritin displays microheterogeneity by resolving into multiple bands upon IEF. Two different interpretations of ferritin IEF bands have been offered. They have been explained as families of hybrid molecules consisting of different proportions of the H and L chains (27) and methodological artifacts (28). Although this controversy suggests that the IEF data should be treated cautiously, the differences between human liver and brain ferritin IEF patterns are striking. Five major and four minor acidic bands were visible for liver ferritin, all clustered in the pI range 4–5.5. In contrast, brain ferritin resolved into about 20 bands spread out over a much greater pI range, with only about 5 bands overlapping those of liver ferritin (Fig. 1 *Right*). The major band was highly positively charged with an apparent pI of 8 and represented 30% of the total protein and iron of the applied sample. This IEF pattern was generally reproducible whether the sample was applied near the anode or cathode, though resolution of all bands was better and the band corresponding to pI of 8 was more intense when the sample was applied near the cathode. A precise matching of the iron content with the individual isoferritin bands was not possible because the bands, though visually quite distinct, could not be adequately separated by the gel slicing procedure. An attempt to analyze the same gel slices for Al was unsuccessful because the minute levels of Al associated with the isoferritins approached the limit of detection and the background contamination of the gel was too high.

The absence of an increased ferritin–Al complex in the livers of Al-fed rats and the presence of high levels of Al bound to the brain ferritin from the control animals is significant. Although the IEF patterns of ferritin from human liver and brain were markedly different, inferences as to the physical significance of such a comparison must be made cautiously. Equilibrium dialysis of human brain, human liver, and horse spleen ferritin against $AlCl_3$ with subsequent Fe, Al, and phosphate determinations showed that the increase in Al bound to ferritin is correlated with an increase in phosphate content. The phosphate/Al ratio was similar for all three ferritins (Table 3). Brain ferritin actually bound less Al than did liver ferritin. This suggests that some explanation other than a structural difference must account for their *in vivo* Al-binding differences. The difference in the total Al bound to the isolated liver and brain ferritins might be due to a difference in the availability of Al to bind to ferritin. In addition to the species difference, the higher Al content of rat brain ferritin compared to human brain ferritin is most probably due to the experimental Al levels to which the rats were exposed. While the binding experiment was performed with mixtures of isoferritins, the possibility of isoferritins differing in their Fe and phosphate content and therefore having differential Al binding properties cannot be excluded.

Table 3. *In vitro* binding of Al to ferritin isolated from human brain, human liver, and horse spleen

Source	Analysis, mol/mol of ferritin			Ratio
	Fe	Al	PO_4	PO_4/Al
Human brain	723	86	353	0.49
Human liver	997	131	421	0.42
Horse spleen	1625	173	833	0.51

The increase in liver ferritin from the Al-fed rats is also a significant finding. Whether these elevated levels are due to a reduction in the turnover of the preexisting ferritin or due to Al-induced increase in the synthesis of new protein remains to be established. While it is well known that Fe induces ferritin synthesis (2), induction by other metal ions has not been demonstrated.

The increase in Al bound to the ferritin from AD patients gives credence to the controversial reports of a correlation between increased Al levels in certain parts of the brain and AD (29). It may be that the increase in Al bound is a reflection of the general increase in the availability of Al to the brains of AD patients.

The isolation of a ferritin–Al complex from brain strengthens the suggested role for ferritin in metal toxicity. Al may be cleared from the liver as a normal process to protect liver enzymes from Al toxicity (30). Therefore, little Al is available for binding to liver ferritin. The increase in Al bound to brain ferritin may be a detoxification mechanism to protect the brain metabolism from Al that cannot be cleared by another mechanism. If, as we suggest, ferritin is a multifunctional molecule (8), the possible alterations in these functions may be influenced by the elevated levels of bound Al.

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