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Aqueous Dissolution of Alzheimer's Disease Aβ Amyloid Deposits by Biometal Depletion*

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Zn(II) and Cu(II) precipitate Aβ *in vitro* into insoluble aggregates that are dissolved by metal chelators. We now report evidence that these biometals also mediate the deposition of Aβ amyloid in Alzheimer's disease, since the solubilization of Aβ from post-mortem brain tissue was significantly increased by the presence of chelators, EGTA, *N,N,N′,N′*-tetrakis(2-pyridyl-methyl) ethylene diamine, and bathocuproine. Efficient extraction of Aβ also required Mg(II) and Ca(II). The chelators were more effective in extracting Aβ from Alzheimer's disease brain tissue than age-matched controls, suggesting that metal ions differentiate the chemical architecture of amyloid in Alzheimer's disease. Agents that specifically chelate copper and zinc ions but preserve Mg(II) and Ca(II) may be of therapeutic value in Alzheimer's disease.

Aβ is the main component of the amyloid deposits that characterize the neuropathologic lesions of Alzheimer's disease (AD).[1] The mechanism leading to the precipitation of this normally soluble protein is unknown, but it is related to the pathogenesis of the disorder, since all mutations linked to familial AD alter Aβ structure or metabolism (1), and the deposition of β-amyloid in the neocortex of transgenic mice overexpressing Aβ is accompanied by most of the other neuropathological features of AD (2). We have previously found that Zn(II), Cu(II), and, to a lesser extent, Fe(III), at low μM concentrations, induce the rapid aggregation of synthetic Aβ (3). These transition metal ions are highly concentrated in the neocortical regions most affected in AD, and all three metal ions are both significantly elevated in the neuropil of these regions in Alzheimer's disease and further concentrated within amyloid plaque deposits (4).

We recently reported that Zn(II)- or Cu(II)-induced Aβ precipitation is reversed by treating the aggregate with metal chelators (5, 6). We hypothesized that if the metal ions within brain amyloid mediated the assembly of Aβ aggregates, then treating tissue with metal chelators should induce the solubilization of Aβ. We tested this hypothesis by extracting Aβ amyloid-bearing post-mortem brain tissue in the presence and absence of various metal ion chelators and assaying the distribution of Aβ within the soluble and insoluble phases.

EXPERIMENTAL PROCEDURES

Tissue Selection—Post-mortem tissues, stored at −80 °C, were obtained from the National Heath and Medical Research Council-supported Brain Bank at the University of Melbourne together with accompanying histopathological and clinical data. AD was assessed according to Consortium to Establish a Register for Alzheimer's Disease criteria (7). In order to examine the chemical architecture of the Aβ deposition that is observed in non-AD aged brain, Aβ immunohistochemistry was used to select age-matched control (AC) cases that did not reach Consortium to Establish a Register for Alzheimer's Disease criteria and in which amyloid deposition, if present, was detectable only in the form of diffuse plaques but not neuritic plaques.

Selection of Chelators—No available chelator is exclusively specific for any particular metal ion; therefore, we surveyed the effects of chelators that display various respective affinities for zinc and/or copper ions relative to more abundant metal ions such as calcium and magnesium. The pK_a values of *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) are as follows: Al(III), negligible; Ca(II), 3; Cu(II), 20.2; Fe(III), 14.4; Mg(II), negligible; Zn(II), 15.4. The pK_a values of EGTA are as follows: Al(III), 13.9; Ca(II), 10.9; Cu(II), 17.6; Fe(III), 11.8; Mg(II), 5.3; Zn(II), 12.6. The pK_a values of bathocuproine (BC) are as follows: Al(III), negligible; Ca(II), negligible; Cu(I), 6.1; Cu(I), 19.1; Fe(III), negligible; Mg(II), negligible; Zn(II), 4.1 (see Ref. 8).

Sample Preparation—The cortical meninges were removed, and gray matter (0.5 g) was homogenized using a DIAX 900 homogenizer (Heidolph & Co, Kelheim, Germany) for three 30-s periods at full speed, with a 30-s rest between strokes, in 3 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4, containing a mixture of protease inhibitors (Bio-Rad), with the exception of EDTA, or in the presence of either various chelators or metal ions prepared in PBS. To obtain the PBS-extractable fraction, the homogenate was centrifuged at 100,000 × *g* for 30 min, and the supernatant was removed and divided into 1-ml aliquots. Protein within a 1-ml supernatant sample was precipitated using 1:5 ice-cold 10% trichloroacetic acid, and pelleted by centrifugation at 10,000 × *g* for 20 min. The pellet was prepared for polyacrylamide gel electrophoresis by boiling for 10 min in Tris-Tricine SDS-sample buffer containing 8% SDS, 10% mercaptoethanol, and 8 M urea. Total Aβ in the cortical samples was obtained by homogenizing in 1 ml of PBS and boiling in sample buffer as above.

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[1] The abbreviations used are: AD, Alzheimer's disease; AC, age-matched control; BC, bathocuproine disulfonic acid; TPEN, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine; PBS, phosphate-buffered saline; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Polyacrylamide Gel Electrophoresis and Western Blotting—Tris-Tricine polyacrylamide gel electrophoresis was performed by loading samples onto 10-well, 10–20% gradient gels (Novex, San Diego, CA), followed by transfer onto 0.2-mm nitrocellulose membrane (Bio-Rad). The Aβ was detected using monoclonal antibodies WO2 (which detects Aβ40 and Aβ42 at an epitope between 5–8), G210 (which is specific for Aβ species that terminate at carboxyl residue 40), or G211 (which is specific for Aβ species that terminate at carboxyl residue 42) (9), in conjunction with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark) and visualized using chemiluminescence (ECL, Amersham Pharmacia Biotech). Each gel included two or more lanes containing known quantities of synthetic Aβ (Keck Laboratory, Yale University, New Haven, CT) as reference standards.

Blot Scanning and Transmission Densitometry Assay for Aβ—Blot films were scanned using a Relisys scanner with transparency adapter (Teco Information Systems, Taiwan), and densitometry was performed using Image 1.6 software (National Institutes of Health, Bethesda, MD). The dynamic range of the film/scanner was determined using a step tablet (catalog no. 911ST600, Eastman Kodak Co.), a calibrated film exposed by the manufacturer to provide steps of known increasing intensity. The quantifiable range of signal intensity for densitometric analysis of our Aβ bands was based on the comparison with a curve obtained by scanning and densitometry of the step tablet. The dynamic range of the scanner was increased by using a transparency adapter rather than reflection.

For the survey comparing levels of Aβ in post-mortem brain samples from AD cases and controls (Fig. 3), the combined signals generated from 4.3-kDa immunoreactive Aβ (apparent monomer) and 8.6-kDa immunoreactive Aβ (apparent dimer) were quantified. Successive ECL exposure times of 2 min, 5 min, 10 min, 15 min, and 30 min were routinely performed to establish the optimal exposure for each individual blot, so that the relative amounts of Aβ measured by transmission densitometry remained in the linear response range of the assay, while determining at what point the signal from the Aβ standards had reached saturating intensity. Preliminary blots were routinely performed to determine how the samples were to be subsequently diluted in order to try to ensure that the Aβ signals fell within the quantifiable portion of the Aβ standard curve. All of the experimental samples extracted from the same brain specimen were initially diluted to the same degree and included on the same blot for analysis (as in Fig. 3*A*). However, it was usually not possible to determine all of the Aβ readings from one blot at one dilution. The Aβ content varied broadly between the extracted samples (note the range in Aβ intensity between the various extracts of the same brain specimens illustrated in the blots in Fig. 3*A*), and therefore it was usually necessary to perform subsequent individual blots on specific samples that had been further diluted in order to generate Aβ signals that fell within the linear range of the standard curve.

This technique was chosen for the Aβ assay in preference to enzyme-linked immunosorbent assay, since it has the advantage of discriminating the M_r of the Aβ immunoreactivity and therefore is less likely to inappropriately detect non-Aβ species such as APP fragments, like those that have recently been found to have been inadvertently cross-reacting with APP in an assay that previously had been considered to be well characterized (10).

The efficiency of the trichloroacetic acid precipitation procedure was validated by testing samples of whole human serum diluted 1:10 to which had been added 2 mg of synthetic Aβ 1–40 or Aβ 1–42. Aβ recovery was assessed by extracting the precipitate into SDS sample buffer and performing Western blot analysis against synthetic Aβ standards as above. Protein in the trichloroacetic acid pellet was estimated by resuspending the pellet in water and assaying the protein recovery using a BCA assay (Pierce). This indicated that the efficiency of protein and Aβ precipitation was approximately 90%. The efficiency of the 8 M urea solubilization was found to be higher and less variable that of formic acid in a parallel, blinded assay conducted independently. All chemicals were obtained from Sigma unless otherwise indicated.

Analysis of Metals—The postcentrifugation pellets were dissolved in 4 ml of 3 N HNO_3 plus 1 N HCl for 24 h and then assayed by inductively coupled plasma atomic emission spectroscopy.

RESULTS

AD frontal cortex was compared with tissue from the same region of AC. A survey of the effects of the chelators at a range of concentrations (0–5 mM) on six AD cases confirmed that the solubilization of Aβ was specifically enhanced by the presence of chelator (Fig. 1), although total trichloroacetic acid-precipitable protein was not affected by any of the chelators at the concentrations tested (data not shown).

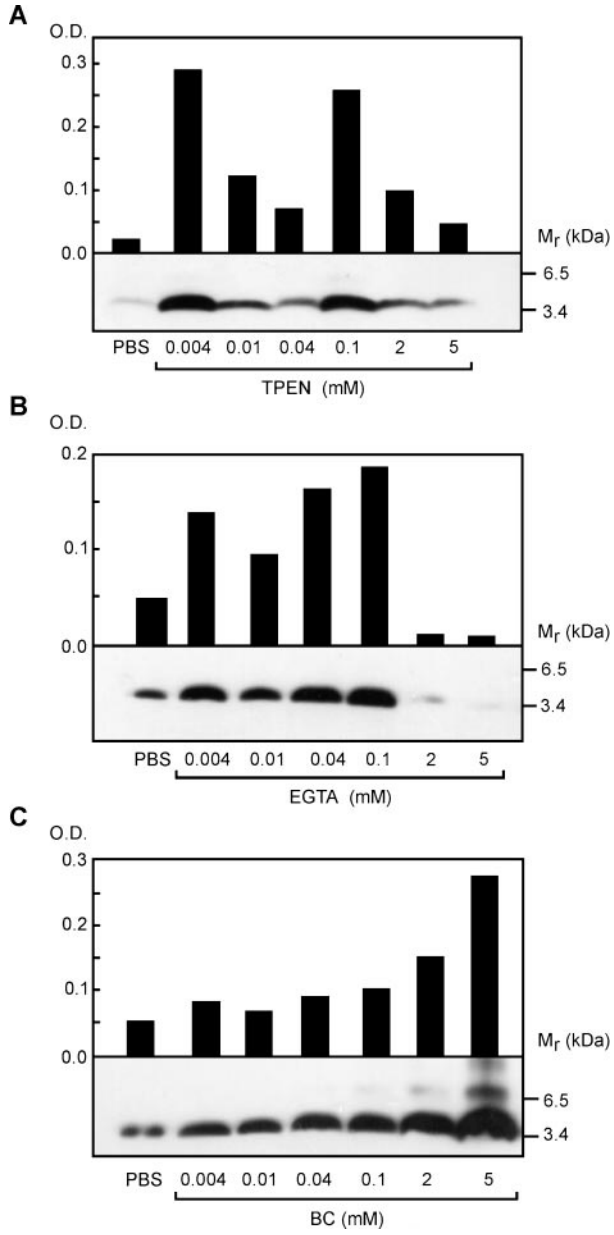

FIG. 1. **Release of Aβ from sedimentable deposits by chelators.** Frontal cortex from an AD brain was homogenized in PBS, pH 7.4, with or without increasing concentrations of TPEN (*A*), EGTA (*B*), or BC (*C*). Following centrifugation, Aβ in the supernatants was visualized by Western blot using anti-Aβ monoclonal antibody WO2 (*lower panels*), and quantified by densitometry (*graphs* above corresponding blots). Although there is considerable variation in the optimum chelator concentration for the maximal recovery of Aβ from case to case, these data are representative of 17 AD cases.

Extraction of AD brain into PBS alone liberated a small amount of Aβ into the soluble phase in every case, confirming previous reports (11–13). In contrast, homogenization in the presence of either EGTA or TPEN at concentrations between 0.004 and 0.1 mM significantly increased soluble Aβ extraction. The optimum concentrations of EGTA or TPEN for the resolubilization of Aβ varied considerably from case to case and did not show linear concentration dependence. Typically, as illustrated in Fig. 1*A*, there was a biphasic response in Aβ extraction as concentrations of EGTA or TPEN were increased. One peak typically occurred when homogenization was performed in the presence of 0.004 mM of either chelator. A second peak in

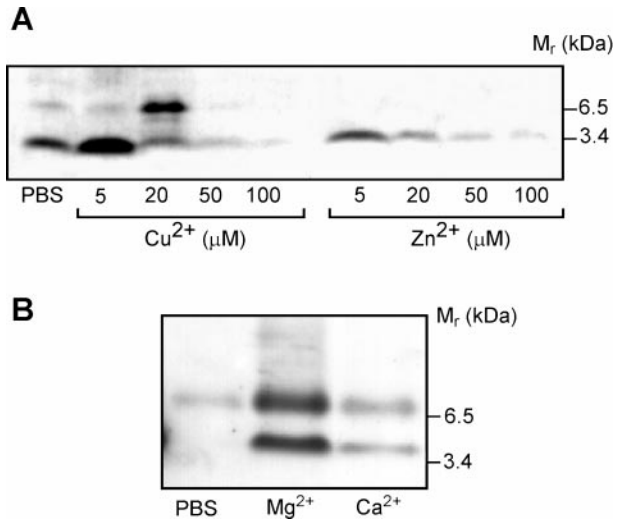

FIG. 2. **The effect of metals upon the solubility of brain-derived Aβ.** *A*, Zn(II) and Cu(II) inhibit the solubilization of Aβ by PBS extraction. Specimens of AD frontal cortex were homogenized in the presence of PBS or varying concentrations of Cu(II) (as sulfate) or Zn(II) (as sulfate). After centrifugation, Aβ in the supernatants was visualized as described in Fig. 1. *B*, Aβ in metal-depleted deposits is liberated into the soluble phase by Mg(II) and Ca(II). Samples of AD frontal cortex were homogenized in 2 mM EGTA, a condition that consistently abolishes the release of Aβ (see Figs. 1 and 3) and removes Zn(II), Cu(II), and other metal ions from the solid phase of the homogenate. After centrifugation, the remaining (metal-depleted) pellets were treated with PBS (pH 7.4) alone, 2 mM Mg(II) in PBS, or 2 mM Ca(II) in PBS and then centrifuged again. Data shown are representative of Aβ in the soluble fraction of the three treated samples, visualized as in Fig. 1.

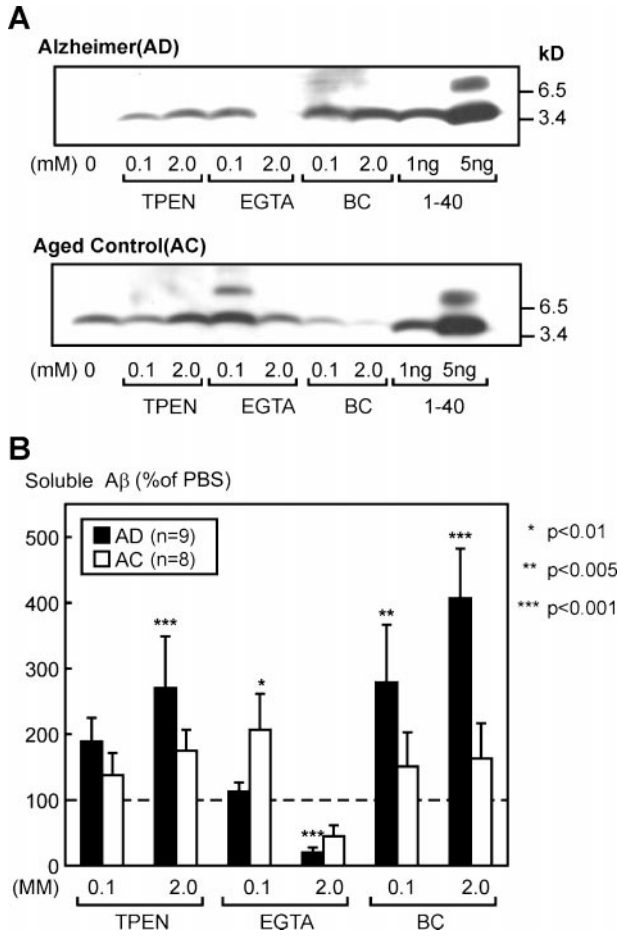

FIG. 3. **Patterns of chelator-mediated release of brain Aβ in AD and age-matched, non-AD tissue.** Post-mortem samples of AD frontal cortex (n = 9) and age-matched controls (n = 8) were treated with PBS, TPEN, EGTA, or BC (chelators at 0.1 and 2 mM), and soluble Aβ was assayed by Western blot. *A*, the soluble material from the seven treatment conditions of each individual case were initially compared on the same blot. An iterative process was used to arrive at the final Aβ concentration for each sample (per g, wet weight) that involved multiple blots quantified by densitometry with reference to two standards of synthetic Aβ1–40 (1 and 5 ng) as well as an 8 M urea extract of the starting tissue. The *upper panel* of *A* shows a representative blot of soluble Aβ extracted from an AD case. The *lower panel* of *A* shows a representative blot of soluble Aβ extracted from a control (AC). For the purposes of the illustration, similar densities of Aβ signal in both the AC and the AD cases shown were achieved by loading more sample onto the AC blot and by slightly prolonging its exposure. However, when normalized against synthetic peptide standards, the amount of Aβ/g of brain sample in typical AC specimens was less than AD (see Table II). *B*, a graphical representation of the effects of chelator-mediated release of brain Aβ derived from data in Table II, which summarizes averaged (±S.E.) data from the AD and AC groups, where the amount of soluble Aβ extracted by the six chelator treatments is expressed as a proportion of the amount of Aβ solubilized by treatment with PBS alone (normalized to 100%) for each individual case.

Aβ soluble extraction occurred at about 0.1 mM for EGTA and 2 mM for TPEN (although there was considerable case-to-case variation, and the case illustrated in Fig. 1*A* had an extraction peak in response to 0.1 mM TPEN). Both TPEN and EGTA were less effective at extracting Aβ when present at concentrations in the millimolar range, and EGTA at ≥2 mM abolished the signal for Aβ (Fig. 1*B*). In contrast, BC elicited a concentration-dependent increase in Aβ extracted from AD tissue (Fig. 1*C*) plateauing at 10 mM. This finding is of interest because BC is highly selective for Cu(I), and the result is compatible with our recent finding that Aβ rapidly binds and reduces Cu(II) to Cu(I) (6, 23), suggesting that a proportion of Aβ assembly is mediated by Cu(I).

Insulin-degrading enzyme, a zinc-metalloproteinase, has been reported to cleave Aβ in the brain and in biological fluids (15). To determine whether chelator-mediated augmentation of Aβ solubilization was due to inhibition of this enzyme, we also performed homogenizations in the presence of 1 mM *N*-ethylmaleimide, a potent inhibitor of insulin-degrading enzyme. Enhancement of Aβ signal was not observed above that of PBS alone (data not shown). To determine whether other enzymatic activities may be artifactually modifying the data, we compared extraction of the brain Aβ at 4 °C to extraction at 37 °C. There was no decrease in Aβ signal to suggest enhanced degradation at the higher temperature. These controls suggest that inhibition of Aβ-cleaving enzymatic activities by chelators does not contribute to the generation of soluble Aβ under these conditions.

To characterize the metal ions participating in the precipitation of brain-derived Aβ and to investigate the nonlinear response of Aβ extraction in the presence of EGTA or TPEN, we added additional metal ions to the extraction system. The presence of Cu(II) or Zn(II) in the PBS homogenization buffer abolished the increased extraction of Aβ caused by chelator treatments (data not shown). Also, the presence of additional Zn(II) (≥5 μM) or Cu(II) (≥50 μM) in the homogenization buffer without chelator abolished extraction of Aβ due to treatment with PBS alone (Fig. 2*A*). Therefore, these metal ions can modulate the solubility of Aβ in this system.

The presence of Cu(II) at 5 μM in the PBS homogenization buffer without chelator increased the extraction of Aβ by PBS (Fig. 2*A*). At pH 7.4, Zn(II) induces far more Aβ aggregation than Cu(II), hence this result may be due to Cu(II) displacing Zn(II) from Aβ. At 20 μM, Cu(II) induces the appearance of an apparent SDS-resistant Aβ dimer, which may be due to an oxidative modification of the peptide or may represent an intermediate produced during the process of Aβ aggregation.

Because millimolar concentrations of TPEN or EGTA unex-

TABLE I

Residual metal levels following treatment of brain homogenates with TPEN

Frontal cortex from AD ($n = 10$) was homogenized in the presence or absence of 0.1 mM TPEN and metal levels in postcentrifugation pellets were determined using inductively coupled plasma atomic emission spectroscopy, normalized for the starting wet weight of the tissue sample. An asterisk indicates significant difference from treatment with PBS alone on t test ($p < 0.01$). Results are shown ±S.E.

	Zinc	Copper	Iron	Calcium	Magnesium	Aluminum
PBS	50.7 ± 4.9	11.9 ± 1.5	227 ± 28.8	202 ± 28.3	197 ± 39.1	44.0 ± 46.2
TPEN	33.2 ± 4.1*	9.8 ± 1.7	239 ± 31.7	210 ± 37.0	230 ± 39.2	65.0 ± 45.0

TABLE II

Concentrations of Aβ extracted by PBS, chelator, and 8 M urea treatment of AD and AC specimens

See legend of Fig. 3. Concentrations of Aβ (in μg/g, normalized for specimen wet weight) extracted from AD and AC specimens by 8 M urea (representing estimated "total" Aβ content), PBS, and two chelators (0.1 and 2.0 mM) were compared. To illustrate the variation, data from each case are shown. Percentage of the total is shown in parentheses.

	AD subject									X ± S.E.
	1	2	3	4	5	6	7	8	9	
Total Aβ	22	77	12	80	15	24	8	33	10	31 ± 9.3
PBS	0.2 (0.9)	1.3 (1.7)	0.1 (0.8)	0.3 (0.4)	0.9 (6.0)	0.5 (2.1)	0.4 (5.0)	1.6 (4.8)	0.6 (6.0)	0.7 ± 0.2 (3.1 ± 0.8)
TPEN, 0.1 mM	0.6 (2.7)	2.8 (1.7)	0.4 (3.4)	0.3 (0.4)	0.8 (5.3)	0.9 (3.8)	0.8 (10)	1.6 (4.8)	0.7 (7.0)	1.0 ± 0.3 (4.3 ± 0.9)
TPEN, 2 mM	0.3 (1.4)	1.8 (2.3)	0.9 (7.5)	0.8 (1.0)	2.0 (13)	1.3 (5.4)	1.2 (15)	2.9 (8.8)	0.5 (5.0)	1.3 ± 0.3 (6.6 ± 1.7)
EGTA, 0.1 mM	0.3 (1.4)	1.5 (1.9)	0.04 (0.3)	0.5 (0.6)	1.1 (7.3)	0.5 (2.1)	0.7 (8.8)	1.7 (5.2)	0.4 (4.0)	0.8 ± 0.2 (3.5 ± 1.0)
EGTA, 2 mM	0.1 (0.5)	0.8 (1.0)	0 (0)	0 (0)	0.2 (1.3)	0 (0)	0.1 (1.3)	0.5 (1.5)	0.1 (1.0)	0.2 ± 0.1 (0.7 ± 0.2)
BC, 0.1 mM	0.14 (0.6)	1.8 (2.3)	0.9 (7.5)	1.4 (1.8)	1.2 (8.0)	0.9 (3.8)	1.6 (20)	3.1 (9.4)	0.5 (5.0)	1.3 ± 0.4 (6.5 ± 1.9)
BC, 2 mM	0.8 (3.6)	3.3 (4.3)	0.8 (6.7)	1.6 (2.0)	1.7 (11.3)	1.5 (6.3)	2.3 (28.8)	3.7 (11.2)	1.5 (15.0)	1.9 ± 0.3 (9.6 ± 2.7)
	AC subject									X ± S.E.
	1	2	3	4	5	6	7	8		
Total Aβ	0.7	0.5	1.0	4.2	2.7	3.2	3.6	0.5		2.1 ± 0.52
PBS	0.17 (24)	0.16 (32)	0.03 (3.0)	0.13 (3.0)	0.18 (6.7)	0.11 (3.4)	0.66 (18.3)	0.06 (12)		0.19 ± 0.07 (12.8 ± 3.9)
TPEN, 0.1 mM	0.12 (17)	0.17 (34)	0.10 (10)	0.29 (6.9)	0.10 (3.7)	0.10 (3.1)	0.60 (16.7)	0.08 (16)		0.19 ± 0.07 (13.4 ± 3.7)
TPEN, 2 mM	0.22 (31)	0.17 (37)	0.10 (10)	0.38 (9.0)	0.26 (9.6)	0.09 (2.8)	1.1 (30)	0.09 (18)		0.30 ± 0.12 (18.4 ± 4.5)
EGTA, 0.1 mM	0.39 (55.7)	0.22 (44)	0.17 (17)	0.28 (6.7)	0.15 (5.5)	0.12 (3.8)	1.0 (27.8)	0.10 (20)		0.30 ± 0.1 (22.6 ± 7.0)
EGTA, 2 mM	0.15 (21.4)	0.03 (6.0)	0.03 (3.0)	0 (0)	0 (0)	0.04 (1.25)	0.2 (5.5)	0 (0)		0.06 ± 0.03 (4.6 ± 2.7)
BC, 0.1 mM	0.09 (12.9)	0.15 (30)	0.15 (15)	0.20 (4.8)	0.18 (6.7)	0.08 (2.5)	0.98 (27.2)	0.08 (16)		0.23 ± 0.11 (14.3 ± 3.7)
BC, 2 mM	0.03 (4.3)	0.04 (8.0)	0.15 (15)	0.24 (5.7)	0.30 (11)	0.08 (2.5)	1.16 (32)	0.10 (20)		0.26 ± 0.14 (12.3 ± 3.5)

pectedly suppressed Aβ resolubilization, we suspected that Mg(II) or Ca(II) may participate in the resolubilization of Aβ. Mg(II) and Ca(II) are more abundant than Cu(II) and Zn(II) in brain samples. Therefore, given the relative affinities of the chelators used, sequestration of Mg(II) and Ca(II) would require higher chelator concentrations than those necessary to complex Zn(II) and Cu(II). Samples of frontal cortex (0.5 g) from AD were homogenized in 2 mM EGTA, a condition that consistently abolishes the solubilization of Aβ (see Fig. 3) while removing Zn(II), Cu(II), and other metal ions from the solid phase of the homogenate. The homogenates were centrifuged at 100,000 × g for 30 min, and the supernatants were discarded. The remaining (metal-depleted) pellets were rehomogenized in a further 2 ml of PBS (pH 7.4) alone, 2 mM $MgCl_2$ in PBS, or 2 mM $CaCl_2$ in PBS, and the homogenates were subjected to centrifugation again at 100,000 × g. Aβ in the soluble fraction was visualized by Western blot with W02 as described. When Mg(II) (2 mM) or Ca(II) (2 mM) was added to the homogenization buffer, there was no appreciable alteration in the extraction of soluble Aβ (data not shown). However, when supplemented to the pellet fraction of a brain homogenate previously depleted of metals by treatment with 2 mM EGTA during homogenization, Mg(II), and to a lesser extent Ca(II), both resolubilized the sedimentable Aβ (Fig. 2*B*). Taken together, these data indicate that although removal of metal ions like Zn(II) and Cu(II) may be necessary for the resolubilization of Aβ deposits, the presence of Mg(II) and Ca(II) is required for the sedimentable Aβ to resolubilize. Therefore, the optimal chelator concentration for the resolubilization of Aβ deposits depends upon an interplay of antagonistic factors, which may explain the nonlinear response of Aβ extraction to increasing chelator concentrations (Fig. 1, *A* and *B*) and the case-to-case variability of the chelator concentrations required to achieve maximal extraction of Aβ.

In order to investigate which metal ions are removed by chelator treatments, we measured the amounts of various metals (aluminum, iron, magnesium, calcium, copper, and zinc) remaining in the brain pellet after treatment with PBS with or without chelator. Analysis of the effects of 0.1 mM TPEN was performed first, since this treatment induced an increase in soluble Aβ in the first six AD samples analyzed and because complete complexation of Mg(II) and Ca(II) was unlikely at that concentration of chelator.

The observed increase in extractable Aβ correlated with significant depletion (30%) in zinc and, to a lesser extent, copper, in each of 10 AD cases examined, when compared with PBS-treated tissue. No other metal measured was significantly influenced by treatment at this concentration (Table I). A survey of the metal content of pellets taken from AD brain homogenates ($n = 2$) treated with the complete range of chelator concentrations described in Fig. 1, confirmed that EGTA treatment at ≥2 mM depleted (>30%) the sample of zinc, calcium, and magnesium, whereas treatment with TPEN at similar concentrations depleted zinc, copper, calcium, and iron. Measurement of metals remaining in the pellet following treatment of these samples over the range of BC concentrations studied indicated that none of the metals was depleted (data not shown). Since BC has an affinity for Cu(I) that is 13 orders of magnitude greater than for Cu(II), the lack of detectable total copper depletion caused by treatment with BC is not unexpected, since copper levels were relatively low in these preparations and the proportion of copper that exists as Cu(I) is likely to be small.

To determine the consistency of chelator effects upon Aβ extraction from brain, we surveyed a larger sample of speci-

mens using two chelator concentrations (0.1 and 2 mM), and also measured the total amount of Aβ in the samples by 8 M urea solubilization. After measuring the effects of treatment with the three chelators upon AD ($n = 9$) and AC ($n = 8$) brain samples, a significant pattern emerged (Fig. 3). For AD cases, significant increases of solubilized Aβ, compared with the baseline amount liberated by PBS treatment, were induced by TPEN at 2 mM (2.7-fold, $p < 0.001$) and BC at 0.1 mM (2.8-fold, $p < 0.005$) and at 2 mM (4.1-fold, $p < 0.001$). The effects of chelators upon the release of Aβ from the AC group were markedly attenuated and therefore did not reach significance with the exception of the effect of 0.1 mM EGTA, which induced a significant increase (2-fold, $p < 0.01$). These data support the possibility that Zn(II) and Cu(I) maintain the aggregated state of Aβ in AD brain but are less important in the architecture of Aβ aggregates in AC. EGTA (2 mM) inhibited the extraction of Aβ in both AD (decreased 80%, $p < 0.001$) and AC (decreased 50%, not significant) groups. This result is compatible with the extraction of Ca(II) and Mg(II) from the tissue homogenates, since these are metal ions that are required for the release of Aβ from deposits that have been depleted of zinc and copper (Fig. 2). The cases analyzed in Fig. 3 were also assayed with reference to the total amount of Aβ extracted from the individual brain specimens (Table II). The concentration of total Aβ in the AD specimens was much greater (31 μg/g) than the total amount in the AC samples (2.1 μg/g). The concentration of Aβ in AD brains that was extracted by PBS alone was 0.7 ± μg/g, representing 3.1% of total Aβ. The amount of Aβ in AD brain extracted by a single treatment with 2 mM BC increased significantly to 1.9 ± μg/g, representing 9.6% (range 2.0–28.8%) of total Aβ. This proportion is likely to be an underestimate of the amount of Aβ that is assembled by biometals, since the result was achieved by exposing the individual brain specimens to only one brief chelator treatment. Repeated extraction cycles resulted in further Aβ release, up to 50% of the starting values. We limited the highest concentration of BC to 2 mM for comparison with other chelators at equimolar concentrations, because our initial data (Fig. 1) indicated that millimolar concentrations of TPEN and EGTA suppressed Aβ solubilization.

Treatment of AD specimens with chelators generated an apparent SDS-resistant Aβ dimer (immunoreactivity migrating at approximately 8.6 kDa) that was not evident when the specimen was treated with PBS alone in over 60% of cases (Fig. 4*A*). Frequently, the appearance of an 8.6-kDa Aβ species was not accompanied by a proportional increase in the amount of apparent Aβ monomer (Fig. 4*A*). These findings are relevant because SDS-resistant dimeric forms of Aβ purified from AD brain have been reported to possess increased neurotoxic properties (16). The possibility that there is a specific metal ion-mediated abnormality of neurotoxic Aβ dimer assembly is being investigated further.

We analyzed Western blots of brain extracts with antibodies that are specific for AβX-40 (G210) and AβX-42 (G211) (Fig. 4*B*), since the latter Aβ subspecies is enriched in AD amyloid plaques (17). We found that treatment with BC significantly increased the solubilization of both Aβ subspecies in AD samples, indicating that AβX-42, while less soluble than the more abundant AβX-40 (18) is nonetheless released by chelation of Cu(I).

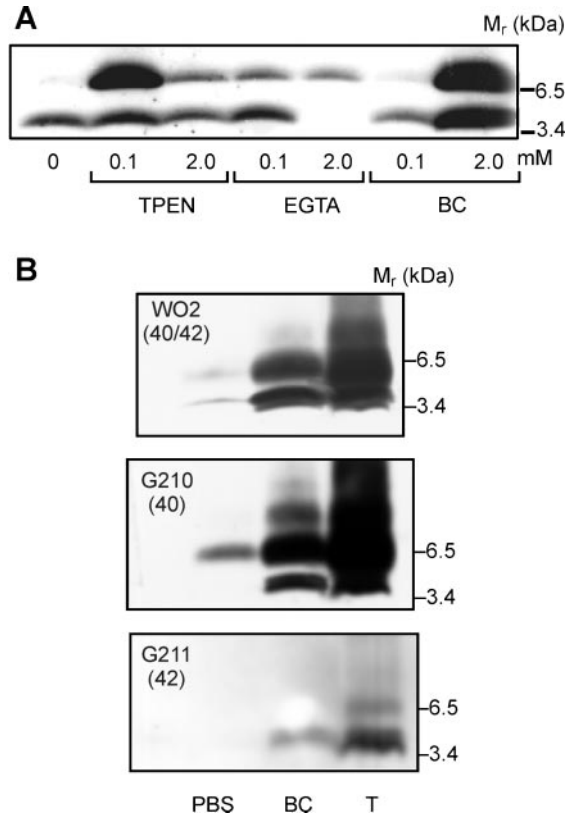

FIG. 4. **Dissection of some components of metal ion-assembled brain Aβ deposits.** *A*, extraction of soluble, SDS-resistant Aβ dimers by chelator treatment. Shown is a representative Western blot of the AD samples that exhibit the release of a soluble Aβ dimer when treated, as in Fig. 3, with 0.1 and 2 mM TPEN, EGTA, or BC. *B*, treatment with chelators promotes the solubilization of Aβ40 and Aβ42 from AD brain tissue. A representative AD specimen was divided and treated with PBS ± 5 mM BC, or 8 M urea to estimate total Aβ content (*T*). Western blots of the extracts were probed with monoclonal antibodies WO2 (raised against residues 5–16, recognizing many Aβ subspecies including Aβ40 and Aβ42), G210 (raised against residues 35–40, recognizing Aβ40), or G211 (raised against residues 35–42, recognizing Aβ42).

DISCUSSION

These data indicate that there is a pool of Aβ within the affected neocortex in AD that is held in sedimentable aggregates by metal ions, likely to be Cu(I) and Zn(II), and that these aggregates are solubilized by treatment with chelators. Mg(II) and Ca(II) were found to be essential for the release of Aβ. The microanatomical site of these collections cannot be determined by our methods, but it is likely to be extracellular, since this is where Aβ deposition in AD is readily demonstrable by morphological techniques and because chelator treatment of AC tissue (possessing much less extracellular plaque deposit) did not release as much Aβ. The possibility of the artifactual combination of cellular metal ions with soluble Aβ leading to Aβ precipitation as a consequence of the tissue homogenization must also be considered. However, since the precipitated fraction of Aβ in AD neocortex is much greater than the soluble cellular pool, this possibility is unlikely to contribute substantially to the phenomenon that we have observed. Other recent observations detecting enrichment of zinc, copper, and iron in amyloid deposits by histological means (4) support the likelihood that our observations reflect the chemical structure of Aβ assembly in amyloid deposits. Aβ-associated, Zn/Cu-metalloproteins apolipoprotein E (19) and α-2-macroglobulin (20–22), may also participate in the reactions we have described.

Our data support the development of chelator compounds as chemotherapeutic agents for AD. One previous clinical trial of a chelator compound, desferrioxamine, was reported to significantly arrest the progression of the disease (14), but no further attempts to reproduce this finding have been reported. Desferrioxamine, like all chelators, is not perfectly specific for a particular metal ion, and although the desferrioxamine trial was thought to target Al(III), it is possible that the beneficial effect of the treatment was due to chelation of Fe(III), Cu(II),

and Zn(II). Our current findings indicate that an ideal therapeutic to dissolve Aβ amyloid would involve a compound that is relatively selective for Cu(I), Zn(II), and possibly Fe(III); that does not sequester Mg(II) or Ca(II); and that coordinates metal ions in the cerebral amyloid mass but not systemically.

We have recently concluded a larger study comparing soluble and insoluble Aβ in AD and AC brains and have found a significant correlation between the PBS-extractable Aβ component and disease severity.[2] Although representing only a small portion of the total Aβ load, an approximate 3-fold difference in the levels of the most readily mobilized Aβ fraction distinguished AD from non-AD in an age-matched population. The present study suggests that 4–7-fold increases in PBS-extractable Aβ can be achieved by direct chelation. At the concentrations used, this effect is observed without apparent impact upon the solubility of other proteins. We have observed that chelator concentrations as low as 4 μM were effective at resolubilizing Aβ deposits from AD brain samples, which indicates that delivering an effective biometal-depleting compound to the amyloid load *in vivo* may not necessitate biologically incompatible doses. Clearly, compounds targeted to the dissolution of aggregated amyloid only have promise as therapeutic agents if the resolubilized and potentially toxic Aβ can be effectively cleared from the AD brain.

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Aqueous Dissolution of Alzheimer's Disease Aβ Amyloid Deposits by Biometal Depletion

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