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Localization of amyloidogenic proteins and sulfated glycosaminoglycans in nontransmissible and transmissible cerebral amyloidoses

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Summary. We report the localization of amyloid β-protein and sulfated glycosaminoglycans in senile plaques and vascular amyloid deposits in brain tissues from patients with Down's syndrome and Alzheimer's disease, and in neurofibrillary tangles of these diseases and those of Guamanian parkinsonism-dementia and amyotrophic lateral sclerosis. We also report the immunolocalization of scrapie amyloid in amyloid plaques containing glycosaminoglycans in kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler's syndrome. Thus, amyloidogenic proteins and sulfated glycosaminoglycans may be copolymerized in amyloid deposits in the nontransmissible and transmissible cerebral amyloidoses.

Key words: Amyloid β-protein – Neurofibrillary tangles – Alzheimer's disease – Scrapie amyloid – Spongiform encephalopathy

The nontransmissible and transmissible cerebral amyloidoses share as a common denominator the deposition of amyloid, an insoluble, proteinaceous substance which exhibits congophilia and green birefringence under polarized light and which consists of fibrils, measuring 10–15 nm in diameter and having a cross β-pleated sheet configuration. The genes encoding the amyloid β-protein precursor of the nontransmissible brain amyloidoses and the scrapie precursor protein of the transmissible cerebral amyloidoses have been cloned and sequenced [3, 9, 12, 22], but the mechanisms by which amyloid is deposited and the relative contributions of components such as glycosaminoglycans are unclear.

We report the localization of amyloid β-protein and sulfated glycosaminoglycans in neurofibrillary tangles of Guamanian parkinsonism dementia and amyotrophic lateral sclerosis. In addition, we confirm the presence of amyloid β-protein in the amyloid deposits in Down's syndrome, Alzheimer's disease and of scrapie amyloid in the plaques of kuru, Creutzfeldt-Jakob disease and Gerstmann-Sträussler's syndrome, as well as the localization of sulfated glycosaminoglycans in these amyloid deposits.

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Materials and methods

We studied formalin-fixed brain tissues from two cases of Guamanian parkinsonism dementia (57-year-old female and 62-year-old male), two cases of amyotrophic lateral sclerosis (47-year-old female and 60-year-old male), two cases of aged Down's syndrome (59-year-old female and 69-year-old male), five cases of Alzheimer's disease (four females, 65, 78, 84 and 87 years and one male, 82 years), two cases of Creutzfeldt-Jakob disease (51-year-old female and 47-year-old male), two cases of Gerstmann-Sträussler's syndrome (40- and 65-year-old males) and one case of kuru (16-year-old male). Tissues from two neurologically normal individuals (50- and 75-year-old females) served as controls. All tissue were stained by heamatoxylin and eosin and by Congo red (Bennhold's method).

Antibodies

Anti-SP43. A 43-amino acid synthetic peptide homologous to amyloid β-protein (SP43), linked to keyhole limpet hemocyanin was used to immunize female BALB/c mice. Immunization and antiserum production has been described elsewhere [22] and modified accordingly. This antiserum did not recognize amyloid deposits found in the transmissible cerebral amyloidoses, including chronic wasting disease in captive mule deer, [7] Rocky Mountain elk and hybrids of captive mule deer and white-tailed deer [8] and in experimental scrapie in mice and hamsters (unpublished data).

Anti-scrapie amyloid. Extracts of brain tissues from terminally ill hamsters infected with scrapie (263K strain) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. The 27- to 30-kDa scrapie amyloid bands were then electroeluted and used to immunize New Zealand white rabbits [1]. Immunization, antiserum production and its characterization have been described elsewhere [1].

Affinity-purified mouse IgG and rabbit IgG (Dako Corporation, Santa Barbara, Calif.) were used as secondary antibodies. Monoclonal antibody to peroxidase-antiperoxidase (PAP) com-

plex (Sternberger-Meyer Immunocytochemicals, Jarrettsville, Md.) and rabbit polyclonal antibody to PAP (Sigma Chemical Co., St. Louis, Mo.) were used as tertiary antibodies. As controls, mouse pre-immune serum, rabbit pre-immune serum, control mouse ascitic fluid (ICN, Lisle, Ill.) and phosphate-buffered saline (PBS) were used instead of the primary antibody.

Immunocytochemistry

The unlabeled secondary antibody PAP technique [21] was used. After deparaffinization, 6-to-8-μm-thick sections were treated with 3 % H_2O_2 and 0.25 % Triton X-100 in PBS (pH 7.4) for 30 min at room temperature to block endogenous peroxidases. Tissue sections from Guamanian patients with amyotrophic lateral sclerosis and parkonsinism-dementia and Down's syndrome were then placed in 50 mM Tris-HCl (pH 7.6), 1 % sodium dodecyl sulfate, 10 mM ethylenediamine tetraacetic acid, 0.07 % 2-mercaptoethanol for 15 min at room temperature, followed by digestion with 10 % pepsin (Sigma) in sodium acetate buffer (pH 4.5) at 37 °C for 30 min. Both steps were followed by washing sectiions in running water and stirring in PBS, 5 min each. Sections were finally treated with 99 % formic acid (Sigma) for 15 min. Sections from other cases were treated with formic acid for 6 min as described elsewhere [10]. To block nonspecific sites, sections were incubated with 6 % bovine serum albumin prepared in PBS for 1 h at room temperature. Incubations with primary antibodies (anti-SP43, diluted 1:1000 for Alzheimer's disease cases and 1:200 for Down's syndrome, Guamanian parkinsonism-dementia and amyotrophic lateral sclerosis cases; anti-scrapie amyloid, diluted 1:100) were conducted overnight at 4 °C in a humidified chamber. Secondary antibodies and antibodies to PAP, diluted 1:20 and 1:100 in PBS, respectively, were placed on tissue sections for 30 min at room temperature. Washes between antibody incubations were done with PBS for 10 min. Color development was achieved with 0.05 % 3, 3′ diaminobenzidine (Sigma) and 0.015 % H_2O_2.

Immunoabsorption

Forty micrograms of a 43-amino acid synthetic peptide homologous to amyloid β-protein was mixed with anti-SP43 (diluted 1:50, 1:200, 1:800, 1:3200 in PBS), incubated at 37 °C for 1 h and spun in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, N.Y.) at 14,000 rpm for 30 min. As controls, SP43 was mixed with a monoclonal antibody to a phosphorylated 200-kDa neurofilament (Sternberger-Meyer Immunocytochemicals) and a set of experiment where SP43 was omitted. Tissue sections were incubated with the corresponding supernatants.

Alcian blue-Critical Electrolyte Concentration (CEC) staining

Differential staining of glycosaminoglycans with Alcian blue was achieved by varying the molarity of magnesium chloride [2, 11, 15]. Both carboxylated and sulfated glycosaminoglycans are stained at 0.1 M $MgCl_2$, while weakly and strongly sulfated glycosaminoglycans, like chondroitin sulfate and heparan sulfate, are stained with Alcian blue at 0.3 M $MgCl_2$. Alcian blue stains only strongly sulfated glycosaminoglycans, like heparan, heparin and keratan sulfates at 0.7 M $MgCl_2$, and at 1.0 M $MgCl_2$ only keratan sulfate is stained [15, 16].

Serial sections were taken into water and allowed to stand in 1 % Alcian blue 8GX (Sigma) prepared in acetate buffer (pH 5.7) and varying concentrations of $MgCl_2$ for 36 h with continuous stirring. Sections were then dehydrated, cleared and mounted.

Results

Histological findings

The neurofibrillary tangles of Guamanian amyotrophic lateral sclerosis and parkinsonism-dementia, Alzheimer's disease and Down's syndrome, as well as senile plaques and cerebral vascular amyloid deposits of Alzheimer's disease and Down's syndrome and amyloid plaques of Creutzfeldt-Jakob disease, Gerstmann-Sträussler's syndrome, and kuru showed congophilia and green birefringence under polarized light.

Immunocytochemical findings

Anti-SP43, a mouse antiserum to amyloid β-protein, did not reveal any senile plaques or cerebrovascular amyloid deposits in brain sections of patients with Guamanian parkinsonism-dementia and amyotrophic lateral sclerosis even after pepsin digestion and formic acid denaturation. However, neurofibrillary tangles in hippocampus and amygdala of Guamanian amyotrophic lateral sclerosis (Fig. 1a) and parkinsonism-dementia (Fig. 1c), respectively, exhibited intense immunoreactivity, partic-

Table 1. Summary of results on Alcian blue-$MgCl_2$ staining and immunostaining of brain tissue sections of nontransmissible and transmissible cerebral amyloidoses

Cerebral amyloidosis	Amyloid deposits	Alcian blue-$MgCl_2$			Immunostaining	
		0.3 M	0.7 M	1.0 M	Amyloid β-protein	Scrapie amyloid
Guamanian amyotrophic lateral sclerosis	Neurofibrillary tangles	++++	+++	–	+++	–
Guamanian parkisonism dementia	Neurofibrillary tangles	++++	+++	+	+++	–
Aged Down's syndrome	Neurofibrillary tangles, senile plaques, cerebrovascular amyloid	++++	+++	+	++++	–
Alzheimer's disease	Neurofibrillary tangles. senile plaques, cerebrovascular amyloid	++++	+++	++	++++	–
Creutzfeldt-Jakob disease	Amyloid plaques	++++	+++	++	–	++++
Gerstmann-Sträussler's syndrome	Amyloid plaques	++++	+++	++	–	++++
Kuru	Amyloid plaques	++++	+++	++	–	++++

Staining was graded as (–) nil, (+) slight, (++) moderate, (+++) intense and (++++) very intense

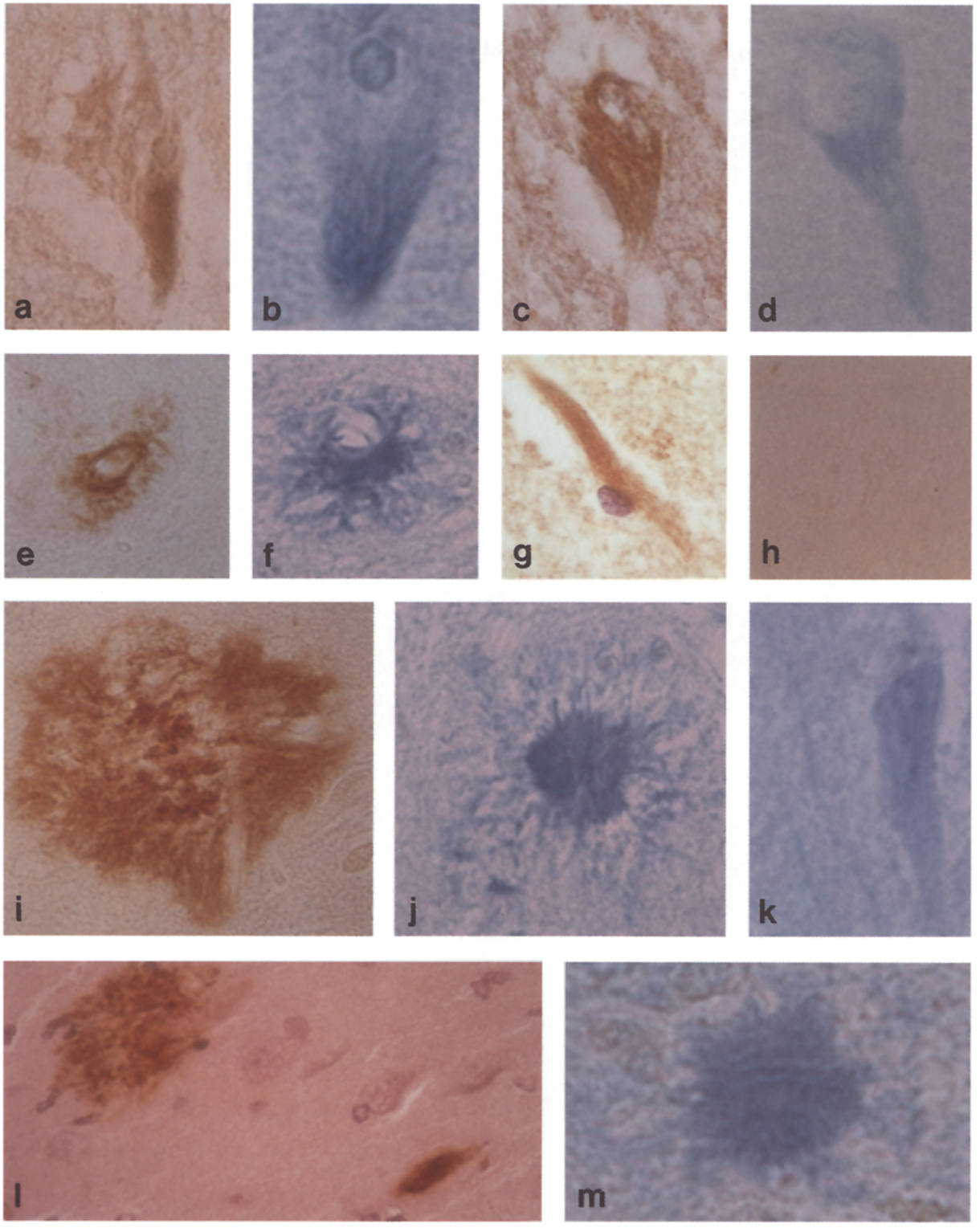

Fig. 1a–m. Immunostaining and Alcian blue-$MgCl_2$ staining of brain sections of nontransmissible cerebral amyloidoses. **a** Anti-SP43-immunoreactive neurofibrillary tangles (NFT); **b** NFT staining with Alcian blue-$MgCl_2$ in hippocampus of Guamanian amyotrophic lateral sclerosis; **c** NFT immunoreactive to anti-SP43; **d** NFT staining with Alcian blue-$MgCl_2$ in amygdala of Guamanian parkinsonism-dementia; **e** cerebrovascular amyloid deposit immunoreactive to anti-SP43; **f** stained with Alcian blue-$MgCl_2$; **g** NFT immunoreactive to anti-SP43; **h** control using mouse pre-immune sera; **i** anti-SP43 immunoreactive amyloid plaque; **j** amyloid plaque stained with Alcian blue-$MgCl_2$, in hippocampus of Down's syndrome. **k** NFT in Down's syndrome stained with Alcian blue-$MgCl_2$; **l** anti-SP43-immunoreactive NFT and amyloid plaque in Alzheimer's disease; **m** amyloid plaque stained with Alcian blue-$MgCl_2$. **a–d, k, m** × 320; **e–j, l** × 128

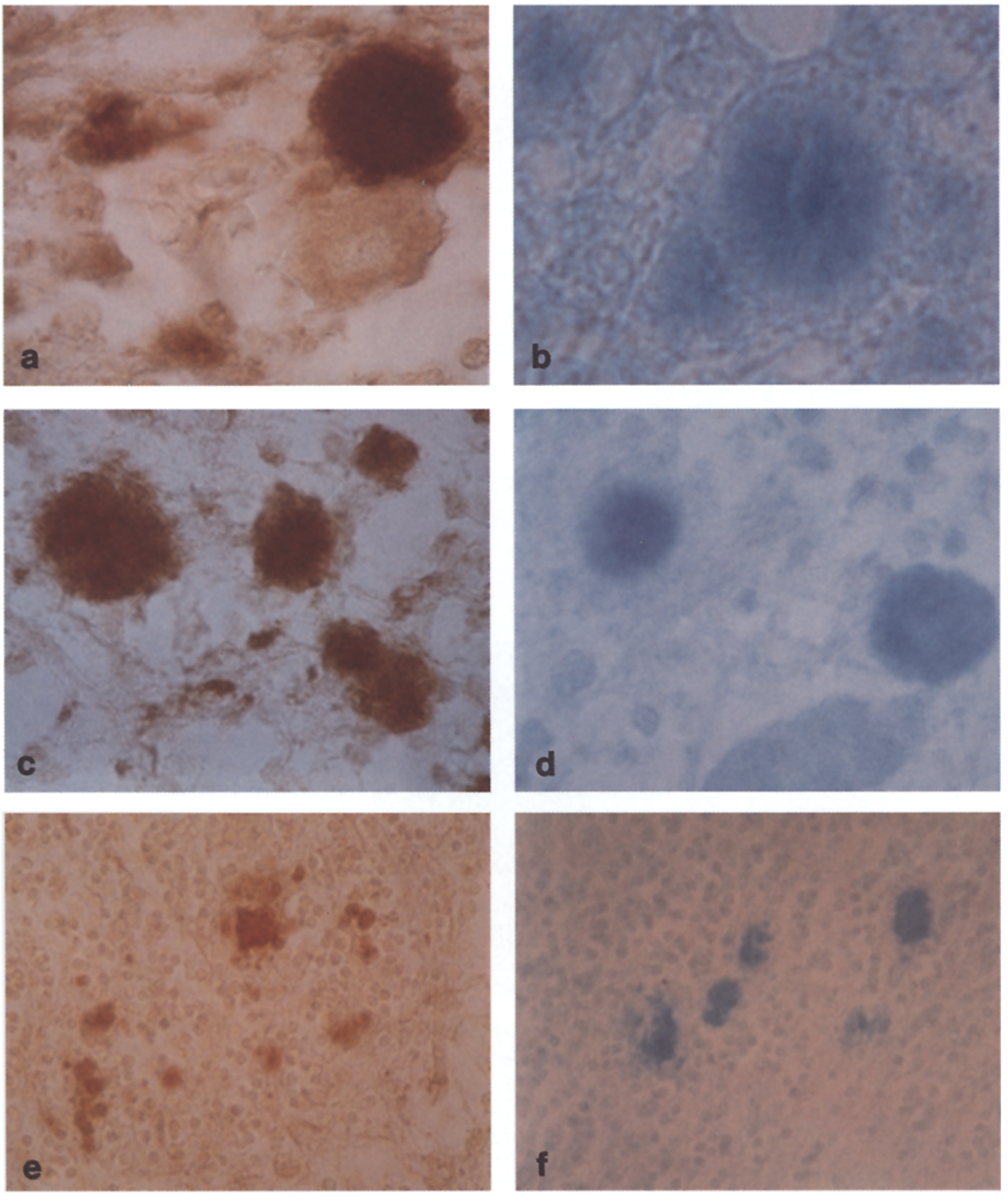

Fig. 2. Immunostaining and Alcian blue-$MgCl_2$ staining of brain sections from transmissible cerebral amyloidoses. Amyloid plaques in cerebellum of kuru (**a, b**), Creutzfeldt-Jakob disease (**c, d**) and Gerstmann-Sträussler's syndrome (**e, f**) exhibiting robust immunoreactivity with antibody against scrapie amyloid (**a, c, e**) and intense staining with Alcian blue containing 0.3 M magnesium chloride (**b, d, f**). **a–d** × 320; **e, f** × 128

ularly on the apical aspect of the tangle or its perikaryal component.

Neurofibrillary tangles (Fig. 1g, l), senile plaques (Fig. 1i, l) and cerebrovascular amyloid deposits (Fig. 1e) in Down's syndrome and Alzheimer's disease and pre-amyloid deposits also demonstrated immunoreactivity to anti-SP43. Amyloid plaques of the transmissible cerebral amyloidosis did not stain with anti-SP43 (Table 1), mouse pre-immune serum, normal mouse ascitic fluid or PBS.

Rabbit antiserum to scrapie amyloid demonstrated immunoreactivity with amyloid plaques in kuru (Fig. 2a), Creutzfeldt-Jakob disease (Fig. 2c), and Gerstmann-Sträussler's syndrome (Fig. 2e), but not with

amyloid deposits in Guamanian amyotrophic lateral sclerosis and parkinsonism-dementia, Down's syndrome and Alzheimer's disease. Rabbit pre-immune serum, normal mouse ascitic fluid and the omission of primary antibodies, as well as the control cases were negative.

Immunoabsorption of anti-SP43 with SP43 abolished immunostaining of amyloid deposits. By contrast, immunostaining with a phosphorylated 200-kDa neurofilament was undiminished after incubation with SP43.

Alcian blue-CEC staining

No differential staining was observed with Alcian blue at 0.1 M $MgCl_2$. However, at 0.3 M and at 0.7 $MgCl_2$, differential staining was found in the neurofibrillary tangles of Guamanian amyotrophic lateral sclerosis (Fig. 1b) and parkinsonism-dementia (Fig. 1d), Down's syndrome (Fig. 1k) and Alzheimer's disease, as well as senile plaques (Fig. 1j, m) and vascular amyloid deposits in Down's syndrome (Fig. 1f) and Alzheimer's disease, amyloid plaques of kuru (Fig. 2b), Creutzfeldt-Jakob disease (Fig. 2d), and Gerstmann-Sträussler's syndrome (Fig. 2f). These findings indicate the presence of weakly and moderately sulfated glycosaminoglycans like chondroitin sulfate and strongly sulfated glycosaminoglycans such as heparan sulfate in these neuropathological structures. Control cases were negative.

Discussion

Our data indicate the presence of amyloid β-protein in the neurofibrillary tangles of Guamanian parkinsonism-dementia and amyotrophic lateral sclerosis, Alzheimer's disease and Down's syndrome and in senile plaques and cerebrovascular amyloid deposits of the latter two conditions. These observations corroborate previous report that the amino acid sequences of intra- and extracellular amyloid deposits share identity [6]. Our observations also confirm the presence of scrapie amyloid in amyloid plaques of kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler's syndrome. Moreover, a common observation is that all of the amyloid deposits described here contain sulfated glycosaminoglycans.

As determined by immunocytochemical techniques, heparan sulfate has been found recently in cerebral amyloid deposits in Alzheimer's disease, Creutzfeldt-Jakob disease, Gerstmann-Sträussler's syndrome and scrapie [18, 20]. Ultrastructurally, glycosaminoglycans are intimately associated with individual fibrils in Alzheimer's disease and experimental amyloidosis [17, 19]. In the latter condition, the deposition of glycosaminoglycans coincide with the deposition of amyloid fibrils in the same anatomical sites in the kidney, liver and spleen [16].

The nature of the interaction between amyloidogenic proteins and glycosaminoglycans is believed to be electrostatic in nature [11, 13, 14]. Glycosaminoglycans may influence the deposition of amyloid proteins should glycosaminoglycans accumulate in the tissue prior to amyloid formation [16]. This phenomenon had been observed in early stages of collagen fibril formation, where the presence of glycosaminoglycans determined the rate and size of fibrils [24].

We have proposed that the mechanism by which amyloid fibrils are deposited in the nontransmissible and transmissible cerebral amyloidoses involves the copolymerization of modified forms of the respective amyloid precursor proteins with glycosaminoglycans [4, 5].

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