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Evidence for Seeding of β-Amyloid by Intracerebral Infusion of Alzheimer Brain Extracts in β-Amyloid Precursor Protein-Transgenic Mice

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Many neurodegenerative diseases are associated with the abnormal sequestration of disease-specific proteins in the brain, but the events that initiate this process remain unclear. To determine whether the deposition of the β-amyloid peptide (Aβ), a key pathological feature of Alzheimer's disease (AD), can be induced *in vivo*, we infused dilute supernatants of autopsy-derived neocortical homogenates from Alzheimer's patients unilaterally into the hippocampus and neocortex of 3-month-old β-amyloid precursor protein (βAPP)-transgenic mice. Up to 4 weeks after the infusion there was no Aβ-deposition in the brain; however, after 5 months, the AD-tissue-injected hemisphere of the transgenic mice had developed profuse Aβ-immunoreactive senile plaques and vascular deposits, some of which were birefringent with Congo Red. There was limited deposition of diffuse Aβ also in the brains of βAPP-transgenic mice infused with tissue from an age-matched, non-AD brain with mild β-amyloidosis, but none in mice receiving extract from a young control case. Aβ deposits also were not found in either vehicle-injected or uninjected transgenic mice or in any nontransgenic mice. The results show that cerebral β-amyloid can be seeded *in vivo* by a single inoculation of dilute AD brain extract, demonstrating a key pathogenic commonality between β-amyloidosis and other neurodegenerative diseases involving abnormal protein polymerization. The paradigm can be used to clarify the conditions that initiate *in vivo* β-amyloidogenesis in the brain and may yield a more authentic animal model of Alzheimer's disease and other neurodegenerative disorders.

Key words: Alzheimer's disease; amyloid; angiopathy; Aβ; transgenic; prion; seeding; neurodegeneration; neuroinflammation; animal model; conformational disease

Similarities in the biophysical properties of amyloidogenic proteins suggest that diseases characterized by abnormal protein deposition share certain etiological mechanisms (Gajdusek, 1994; Olafsson et al., 1996; Kisilevsky and Fraser, 1997; Lansbury, 1997; Price et al., 1998; Prusiner, 1998; Trojanowski and Lee, 1998; Wakabayashi et al., 1998; Koo et al., 1999; Vidal et al., 1999). The significance of the protein deposits per se for the pathogenesis of the diseases is debatable, but the universal tendency of the offending proteins to self-aggregate suggests that ordered protein polymerization is important in the pathogenesis of these disorders. The polymerization of the β-amyloid peptide (Aβ) into senile plaques and cerebrovascular amyloid is a central feature of cerebral β-amyloidoses, such as Alzheimer's disease (AD) (Hardy et al., 1998; Selkoe, 1999). Although the seeded polymerization of Aβ can be achieved *in vitro* (Harper and Lansbury, 1997), inducing the deposition of Aβ *in vivo* has been an elusive goal (Prusiner, 1985; Brown and Gajdusek, 1991). Attempts to produce AD-like pathology by intracerebral infusion of AD tissues into animals have produced inconsistent and sometimes paradoxical results (Goudsmit et al., 1980; Manuelidis and Manuelidis, 1991; Godec et al., 1994). Marmosets injected with AD brain homogenates developed scattered deposits of Aβ in the neuroparenchyma and cerebral vasculature 6–7 years after inoculation (Baker et al., 1994). However, the resultant amyloid lesions were not preferentially localized to the injection sites, and the long incubation period limits the utility of the paradigm.

The ordered aggregation of fibrillogenic proteins into amyloid is most efficient above a critical protein concentration (Harper and Lansbury, 1997). In transgenic mice overexpressing the prion protein (PrP) gene, the efficiency of prion disease transmission is enhanced by increasing PrP expression levels (Prusiner et al., 1990). Mice transgenic for the human β-amyloid precursor protein (βAPP) gene (Tg[HuAPP695.K670N-M671L]2576) (Hsiao et al., 1996) constitutively express excess human βAPP in brain; as they age, the mice sequester increasing quantities of the amyloidogenic Aβ peptide intracerebrally, and they begin to develop senile plaques at approximately the age of 9 months (Hsiao et al., 1997). We therefore reasoned that βAPP-transgenic mice would be an advantageous model for testing the hypothesis that β-amyloidosis can be induced by the intracerebral injection of an appropriate agent. Because the necessary components of an effective, *in vivo* Aβ-seeding agent are unknown, we chose to infuse extracts from AD brains, which unquestionably manifest the necessary conditions for β-amyloidogenesis. Our results show that β-amyloid deposition can be prematurely induced in βAPP-transgenic mice, but not in nontransgenic controls, by the intracerebral infusion of dilute AD brain extract.

Preliminary results from this study were presented at the conference on Vascular Factors in Alzheimer's Disease, Newcastle, England, May 27, 1999 (Walker et al., 1999).

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Table 1. Autopsy cases from which cortical extracts were prepared

Case	Age[a]	PMI[b]	AD	ApoE	Sex	1–40[c]	1–42[c]
AD case 1	81	2.75	Yes	3/4	F	11.1	1.4
AD case 2	84	4.3	Yes	4/4	M	8.6	3.1
AD case 3	91	4.0	Yes	3/4	F	8.8	2.7
AD case 4	84	2.3	Yes	3/4	F	9.8	2.2
Control 1	77	2.5	No	2/3	F	2.4	0.4
Control 2	25	7.0	No	3/3	M	2.0	BDL

[a]Years of age at time of death.

[b]Postmortem interval (hours).

[c]Aβ1–40 and Aβ1–42 (ng/ml) in 1% extract.

BDL, Below detection limit.

MATERIALS AND METHODS

Human tissue extracts. Tissue for preparation of extracts was derived at autopsy from the superior frontal gyrus or lateral orbital cortex of four people who had died of confirmed Alzheimer's disease and from two neurologically normal controls (Table 1). Neuropathological examination revealed profuse senile plaques and neurofibrillary tangles in the neocortex and hippocampus of all four Alzheimer's subjects that fulfilled the Consortium for the Establishment of a Registry for Alzheimer's Disease (CERAD) criteria for AD. The aged control case was a nondemented female who died at the age of 77 years of chronic obstructive pulmonary disease and emphysema. Neuropathologically, the neocortex contained scattered diffuse, Aβ-immunoreactive senile plaques; there were no neurofibrillary tangles in the neocortex or hippocampus, and this person did not fulfill the CERAD criteria for AD. The young control case was a 25-yr-old male who died of cardiac trauma and who had no AD-like pathology in the brain.

The tissue samples were fresh-frozen on dry ice and stored at −80°C. They were homogenized at 10% (w/v) in sterile HBSS, vortexed for 2 min, probe-sonicated for 3 sec, revortexed, and centrifuged at 3000 × *g* for 5 min to remove tissue debris, including blood vessels and plaque cores. The supernatant was recovered and immediately frozen (−80°C). The frozen supernatant was then thawed and further diluted 1:10 (v/v, in HBSS) to a final concentration of 1% immediately before surgical injection. ELISA of Aβ levels in the injected material revealed high levels of both Aβ40 and Aβ42 in the AD cases, intermediate levels in the aged control case, and low or undetectable levels in the young control case (Table 1).

Subjects and experimental design. In the long-term incubation study (Table 2), 46 3-month-old male mice (34 Tg2576 and 12 nontransgenic littermate controls) were studied: (1) AD-brain-injected (AD cases 1–4), (2) aged control brain-injected, (3) young control brain-injected, (4) vehicle-injected, and (5) untreated. These mice survived for 5 months, at which age (8 months) amyloid deposition normally is not found in Tg2576 mice (Hsiao et al., 1997). In the short-term incubation study, six additional male transgenic mice were injected with brain extract from AD case 1 to assess the early pathological changes at 5 d, 2 weeks, or 4 weeks after inoculation. Three of the 46 long-term mice and one short-term animal died during the incubation period (three mice injected with AD extract and one with aged control extract). These mice were not included in the analysis. All surviving mice were monitored weekly in their cages for signs of behavioral changes. In addition, a subset of 22 mice representing the long-term treatment groups were tested for spatial memory in the Morris water maze immediately before killing.

Stereotaxic surgery. Stereotaxic injections were made under sodium pentobarbital anesthesia (60 mg/kg, i.p.). Bregma and the skull surface served as the stereotaxic zero points (Franklin and Paxinos, 1997). The dura mater was surgically exposed, a 27 gauge cannula was lowered into the right hippocampus [anterior (A) −2.0, lateral (L) −1.3, ventral (V) −2.2], and 3.5 μl of clear, 1% brain extract or HBSS was slowly infused (1.0 μl/min) at this site. The cannula was then raised 1.4 mm (A −2.0, L −1.3, V −0.8), and an additional 1.5 μl of infusate was injected into the overlying neocortex. After the cortical injection, the cannula was left in place for 2 min before withdrawal. Postoperatively, the mice were maintained on a warming pad until they had recovered from the anesthesia, after which they were returned to their cages. All procedures were conducted in accordance with institutional guidelines for the care and use of experimental animals.

Table 2. Number of transgenic (N_{TG}) and wild-type (N_{WT}) mice analyzed in the 5 month treatment groups

Treatment	N_{TG}	$Seeding_{TG}$	N_{WT}	$Seeding_{WT}$
AD case 1	5[a,b]	Yes	3	No
AD case 2	5	Yes	—	—
AD case 3	4[a]	Yes	—	—
AD case 4	5	Yes	—	—
Aged control	2[a]	Slight	3	No
Young control	4	No	—	—
Sham (HBSS)	3	No	3	No
Untreated	3	No	3	No
Total mice	31		12	

[a]One mouse in each of these groups died during the 5 month incubation period; the numbers shown indicate the mice that remained for analysis.

[b]These five mice represent two separate experiments. Three mice were injected in the initial study (one died during incubation); 6 months later, an additional three mice were injected with extract from the same case. All five mice showed similar levels of extract-induced Aβ-immunoreactivity.

Histology and quantitation of Aβ deposits. At the age of 8 months (5 months after surgery), the 43 mice remaining in the long-term study were killed under deep sodium pentobarbital anesthesia; the five AD tissue-injected mice for short-term analysis were similarly killed at 5 d ($n = 1$), 2 weeks ($n = 2$), and 4 weeks ($n = 2$) after surgery. Mice were perfused transcardially with PBS, pH 7.4, followed by phosphate-buffered 4% paraformaldehyde, pH 7.2. The brains were post-fixed in this solution for 24 hr, then cryoprotected in phosphate-buffered 25% sucrose, pH 7.4, frozen on dry ice, and coronally sectioned at 20 μm thickness. Tissue sections were stained immunohistochemically using primary monoclonal antibodies 6E10 (Senetek, Maryland Heights, MO) to amino acids 5–14 of Aβ; 4G8 (Senetek) to amino acids 17–24 of Aβ; AT-8 (Polymed, Chicago, IL) to phosphorylated tau; with a monoclonal antibody to glial fibrillary acidic protein (GFAP; Boehringer Mannheim, Indianapolis, IN); and with polyclonal antibodies R163 to the C-terminal 8 amino acids of Aβ40, and R165 to the C-terminal 8 amino acids of Aβ42 (Pankaj Mehta, Institute for Basic Research in Developmental Disabilities, Staten Island, NY). Additional sections were stained with Congo Red and viewed under cross-polarized light, or with hematoxylin and eosin.

The deposition of Aβ was quantified in the hippocampus at the coronal level of the injection site by point-counting analysis of the area occupied by Aβ immunoreactivity (antibodies 6E10 and 4G8) as a function of the total hippocampal area. Differences in Aβ load were evaluated statistically by *t* test or ANOVA with two-tailed significance thresholds.

RESULTS

Intracerebral injection of brain extract from all four AD cases into β APP-transgenic mice induced the deposition of Aβ peptide in the ipsilateral hippocampus and (to a lesser degree) neocortex after a 5 month incubation period. Aβ deposits were distributed throughout the hippocampus, but tended to accumulate prefer-

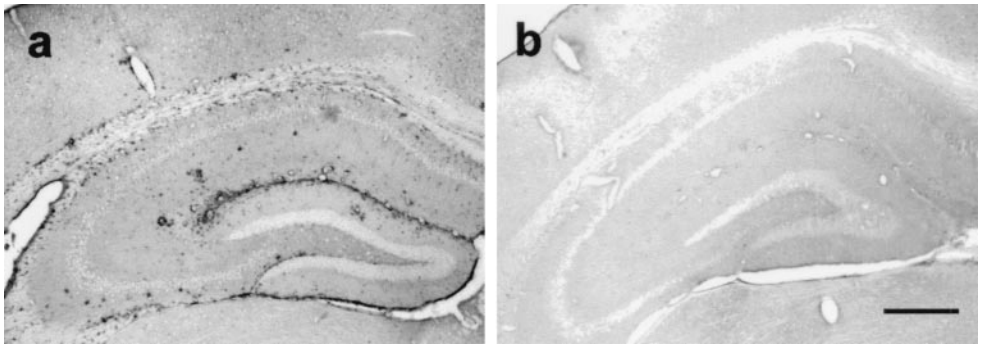

Figure 1. Eight-month-old Tg2576 (*a*) and nontransgenic (*b*) mice that had received equivalent intracerebral injections of dilute AD brain extract (case 1) 5 months earlier. *a,* Aβ immunoreactivity in the hippocampus of a transgenic mouse injected with AD brain extract. Note especially the profuse Aβ deposition along the hippocampal fissure. *b,* Absence of Aβ immunoreactivity in a nontransgenic, littermate control mouse injected with AD brain extract. Antibody 4G8. Scale bar, 500 μm.

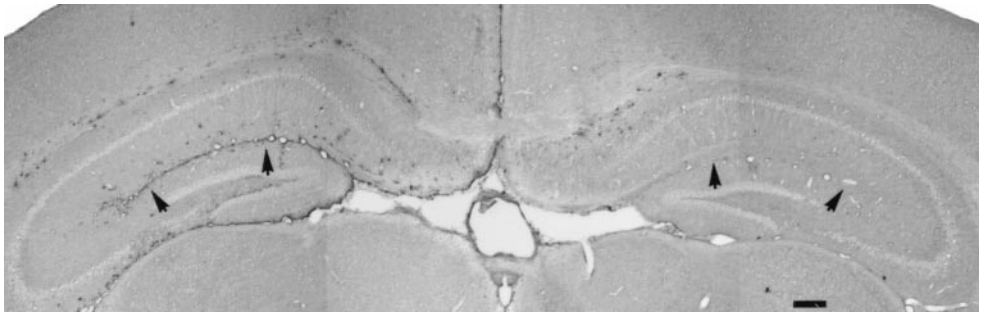

Figure 2. Photomontage of a coronal section through the dorsal forebrain of a transgenic mouse that had been infused unilaterally (*left side*) with AD brain extract (AD case 3). A limited amount of Aβ is deposited in and around the corpus callosum and also in the medial aspect of the contralateral hemisphere. *Arrows* mark the hippocampal fissure in each hemisphere. Antibody R165 to Aβ42. Scale bar, 200 μm.

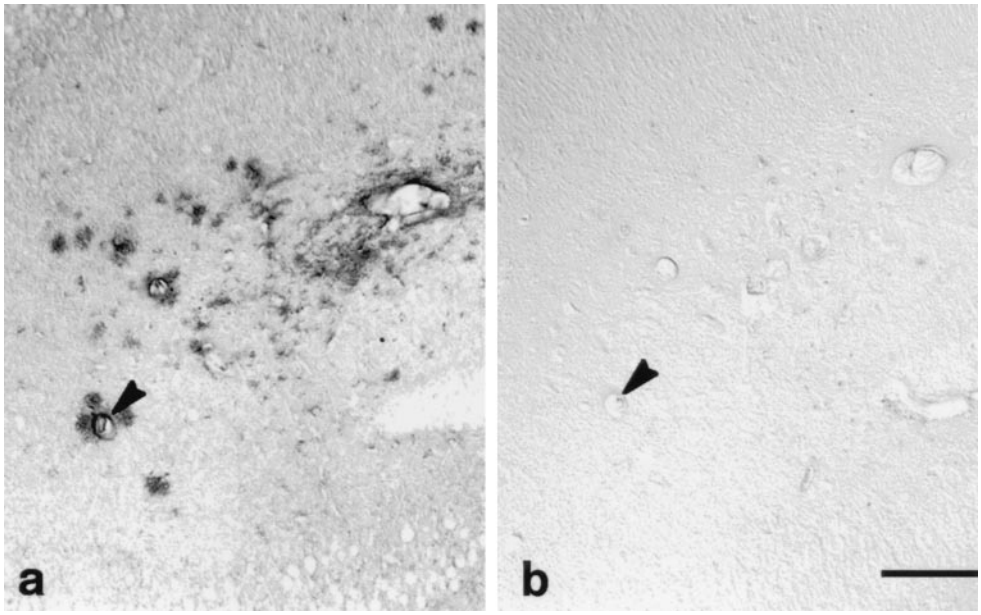

Figure 3. Antibody–antigen adsorption control. *a,* Aβ immunoreactivity in the hippocampus of a Tg2576 mouse that had been injected with AD brain extract (AD case 1; antibody 6E10). *b,* Adjacent control section in which antibody 6E10 was preadsorbed with Aβ1–28 peptide. The *arrowheads* denote the same blood vessel that has been transversely sectioned in *a* and *b*. Scale bar, 100 μm.

entially along the hippocampal fissure, around blood vessels, and beneath pial surfaces (Figs. 1–4). Although concentrated most heavily in the injected structures, some Aβ deposits emerged in regions well beyond the injection sites, even extending along the corpus callosum and into the contralateral hemisphere of several mice (Fig. 2). Aβ immunoreactivity in the noninjected hippocampus was ~10% of that on the injected side, and this asymmetry was statistically significant ($t_{(18)} = 5.19$; $p < 0.01$).

Aβ immunoreactivity was completely blocked by preadsorption of antibody 6E10 with Aβ1–28 peptide (Fig. 3). There was no evidence of Aβ immunoreactivity in 3- to 4-month-old transgenic mice killed 5 d, 2 weeks, or 4 weeks after AD tissue injection, indicating that the immunoreactivity present at 8 months was not the injectate itself. Neither AD nor control brain extracts produced Aβ deposition in nontransgenic control mice (Fig. 1*b*), nor were Aβ deposits seen in the brains of transgenic mice that were uninjected or vehicle-injected. Quantitative analysis confirmed that the transgenic/AD tissue-injected mice had significantly more Aβ deposition in the infused hippocampus than did the other groups of 8-month-old transgenic mice (Fig. 4). Injection of brain extract from a young human resulted in no seeding of senile plaques or cerebrovascular amyloid in any mice (Fig. 4*d*). Transgenic mice injected with cerebral tissue extract from an aged, non-AD case displayed a small amount of Aβ immunoreactivity in the injected hemisphere (Fig. 4*c*) with a distribution similar to that in the AD extract-injected mice, except that the Aβ accumulation within the brain was only 10–15% of that seen in AD extract-injected transgenic mice (Fig. 4*e*).

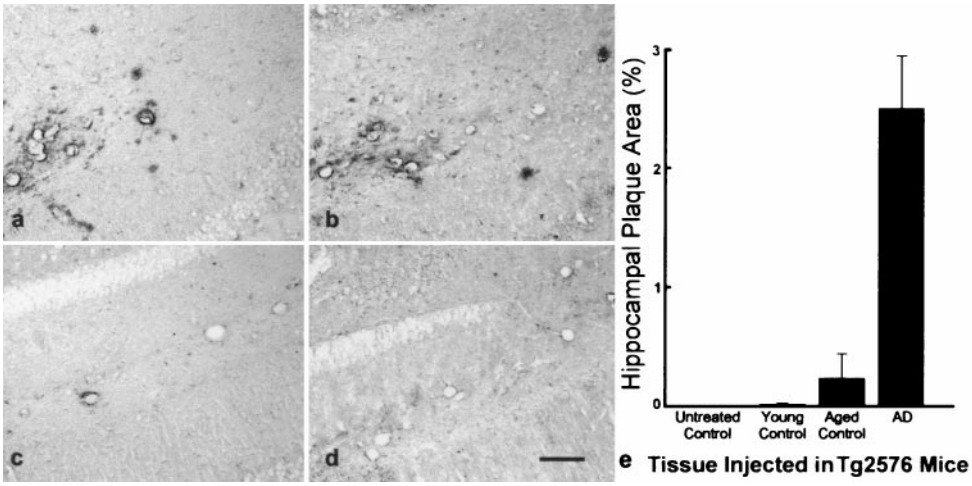

Figure 4. Aβ immunostaining of the hippocampus in two Tg2576 mice injected with AD brain extract (*a, b*) and in two transgenic mice injected with control brain extracts (*c, d*). *e* shows the amount of hippocampal Aβ deposition in Tg2576 mice in the four long-term experimental groups. *a,* Tg2576 mouse injected with extract from AD case 1. *b,* Tg2576 mouse injected with extract from AD case 2. *c,* Tg2576 mouse injected with extract from an aged, non-AD case. *d,* Tg2576 mouse injected with extract from a young control case. Antibody 6E10. Scale bar, 100 μm. *e,* Mean (± SEM) hippocampal plaque area occupied by Aβ-immunoreactive deposits in all AD-tissue-injected Tg2576 mice compared to untreated control transgenic mice (sham and unoperated), as well as mice infused intracerebrally with young control and aged control brain extracts. The treatment effect was statistically significant ($F_{(3,27)} = 6.02$; $p < 0.01$). Antibody 4G8 (antibody 6E10 yielded similar results).

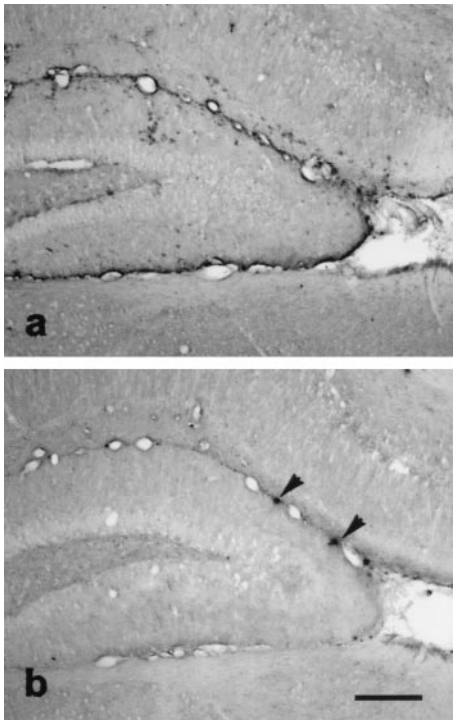

Figure 5. Comparative immunostaining of Aβ42 (*a*) and Aβ40 (*b*) in a Tg2576 mouse infused with extract from AD case 3. Aβ40 deposits were infrequent in AD tissue-infused animals at 8 months of age, and when they occurred they were usually small, compact parenchymal lesions (*arrowheads*) or cerebrovascular deposits. Scale bar, 200 μm.

In contrast to the deposits that appear normally in Tg2576 mice after 9 months of age, the seeded hippocampal deposits in 8-month-old, AD extract-injected mice were mostly diffuse in appearance (Figs. 3, 4); they were strongly immunoreactive with an antibody to Aβ42 (Fig. 5*a*), infrequently stained with an antibody to Aβ40 (Fig. 5*b*), and numerous blood vessels were affected, especially along the hippocampal fissure (Figs. 3, 4). A small number of plaques and blood vessels in AD tissue-injected transgenic mice were birefringent after staining with Congo Red (Fig. 6); when the same deposits could be identified in adjacent sections, the congophilic amyloid was usually Aβ40-immunopositive.

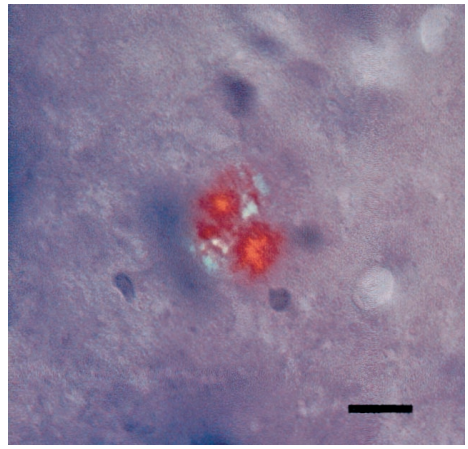

Figure 6. Congo Red-stained amyloid plaque in the hippocampus of a Tg2576 mouse infused with extract from AD case 1. Crossed polarizing filters. Scale bar, 10 μm.

There was no evidence of tau hyperphosphorylation or spongiform change in any mice; obvious gliosis and neuronal loss were only evident in the tissue directly damaged by the injection. The mice showed no signs of motoric or other behavioral dysfunction throughout the postsurgical incubation period; the performance of extract-injected mice tested in the water maze did not differ significantly from that of control mice. Although the injection produced an acute inflammatory reaction directly after surgery, signs of frank inflammation were minimal at 5 months after injection.

DISCUSSION

Our findings show that Aβ deposition can be induced in βAPP-transgenic mice by the intracerebral infusion of dilute extracts of human neocortex. Thus, some factors in the human brain promote the polymerization of Aβ, and our results indicate that the necessary substances are especially plentiful in the Alzheimeric brain. Induction of Aβ was achieved using tissue from four separate AD cases and was replicated in a separate experiment using the initial AD case. To minimize the possibility that the Aβ-immunoreactive material in the brains of extract-infused Tg2576 mice was introduced in the injectate itself, the homoge-

nates were centrifuged to remove plaque cores, blood vessels, and other debris from the supernatant. As a result, there was no Aβ immunoreactivity in nontransgenic mice injected with human tissue extract or in transgenic mice 5 d, 2 weeks, or 4 weeks after injection. The absence of Aβ deposits in the 4 weeks after infusion indicates that a lag phase precedes the precipitation of Aβ peptide in the brains of extract-injected transgenic mice.

Under normal circumstances, Tg2576 mice begin to develop β-amyloid deposits at ~9 months of age (Hsiao et al., 1997), so we assessed Aβ induction between 3 and 8 months of age, before the typical onset of age-related β-amyloidosis. It is significant that we detected Aβ deposits only in 8-month-old transgenic mice that were injected with aged human brain extracts and not in age-matched transgenic mice injected with young brain extract or in untreated transgenic mice. In addition, Aβ deposits in transgenic mice were 10-fold more abundant in the injected hippocampus than in the contralateral structure. Finally, although traumatic brain injury has been shown to acutely increase cerebral Aβ peptide levels in βAPP (PDAPP)-transgenic mice (Smith et al., 1998), our vehicle-injected transgenic mice were devoid of Aβ immunoreactivity, indicating that surgical trauma alone is insufficient to induce β-amyloidosis in these mice. Interestingly, the Aβ deposits in extract-injected mice were mostly diffuse, Aβ42-immunoreactive deposits; typically, before 12 months of age, Tg2576 mice develop discrete, generally dense deposits, 90% of which are immunoreactive for both Aβ40 and Aβ42 (Frautschy et al., 1998).

We speculate that a nucleating seed for the ordered aggregation of Aβ is abundant in the AD brain, and this seed, in the context of high levels of endogenous Aβ in βAPP-transgenic mice, stimulates the polymerization of the peptide into senile plaques and cerebrovascular amyloid. The nature of the seeding agent remains to be determined, and it is possible that multiple factors must interact for efficient seeding to occur. Previous studies in rats have demonstrated that intracerebrally injected, purified senile plaque cores tend to be concentrated at the site of injection or are carried by phagocytic macrophages to the abluminal side of nearby blood vessels (Frautschy et al., 1992). Perivascular Aβ immunoreactivity was prominent in our transgenic mice as well, but the great majority of vessels were not congophilic, and, with only a few exceptions, they contained exclusively Aβ42. Furthermore, in our model, a lag phase precedes Aβ deposition. The chronic infusion of soluble, synthetic Aβ into nontransgenic rodent brains does not result in amyloid deposits similar to those observed in the AD brain (Frautschy et al., 1996), although the inclusion of specific cofactors can enhance deposition (Snow et al., 1994; Frautschy et al., 1996), suggesting that other molecules are important for the intracerebral generation of fibrillar deposits. The seeding model will enable the determination of the requisite elements, including possible molecular chaperones, that initiate Aβ polymerization *in vivo*.

A growing body of evidence implicates the aggregation of misfolded proteins in the pathogenesis of amyloidoses, spongiform encephalopathies, and other neurodegenerative diseases in which protein accumulation is a common feature (Gajdusek, 1994; Olafsson et al., 1996; Carrell and Lomas, 1997; Kisilevsky and Fraser, 1997; Lansbury, 1997; Price et al., 1998; Prusiner, 1998; Trojanowski and Lee, 1998; Wakabayashi et al., 1998; Koo et al., 1999; Vidal et al., 1999). In the case of β-amyloidosis, the highly fibrillogenic Aβ42 may be culpable (Younkin, 1995; Harper and Lansbury, 1997). If Aβ itself is the seeding factor in the AD brain fractions, it will be instructive to establish which species of Aβ is most effective, what cofactors are required, and whether the AD brain contains a conformational variant of Aβ that is especially potent at seeding the polymerization of the endogenous peptide. Injection of synthetic Aβ40 and Aβ42, along with potential cofactors such as apolipoproteins or extracellular matrix proteins, could pinpoint the necessary ingredients for optimal seeding of Aβ, provided that problems of synthetic Aβ batch variation and peptide aging (Price et al., 1992; Findeis and Molineaux, 1999) can be satisfactorily resolved. Coinjection of synthetic Aβ with extracts from young brains also would help to establish whether the necessary chaperones for seeding are present only in the aged brain. In addition, the possibility that metal ions (Atwood et al., 1998) may modulate the polymerization of Aβ *in vivo* warrants further exploration. The role of apolipoprotein E (ApoE) type in initiating β-amyloid deposition also remains an open question. All of the AD cases that we studied happened to bear one or more ApoEε4 alleles, but the two control cases had none. ApoE4 lowers the average age of the onset of β-amyloidogenesis in humans (Walker et al., 2000); further studies are needed to determine why this is so, and whether ApoE type might affect the ability of AD tissue extracts to seed amyloidosis in transgenic mice.

In elderly, nondemented humans, diffuse plaques containing only Aβ42 tend to arise, on average, at least a decade before dense-cored plaques containing Aβ40 emerge (Walker et al., 2000). In contrast, the first Aβ deposits to appear in normal, aged Tg2576 mice tend to be small, compact, and immunopositive for both Aβ40 and Aβ42 (Frautschy et al., 1998). The preponderance of diffuse Aβ42 deposits in extract-injected βAPP-transgenic mice suggests that Aβ42 is the initially seeded form of the peptide and that the addition of Aβ40 to the lesions is a later event. Thus, βAPP-transgenic mice infused intracerebrally with AD tissue may model more faithfully the early stages of amyloid deposition in humans than do normal, aged Tg2576 mice. It will be informative to assess the evolution of seeded Aβ deposits in βAPP-transgenic mice as the animals progress beyond 8 months of age. Human brain extracts might also be used to seed protein deposition in animal models of other neurodegenerative disorders, for example in accelerating tauopathy in tau-transgenic mice (Ishihara et al., 1999).

Although there is little indication of chronic inflammation in the injected mice, the participation of an infectious microorganism in promoting the amyloidosis cannot yet be definitively ruled out. Furthermore, aside from amyloid deposition, the injected mice did not develop other hallmarks of AD, such as neurofibrillary tangles or neuronal loss, during the 5 month incubation period studied. However, demonstration that β-amyloid formation can be induced in βAPP-transgenic mice may help to illuminate the early stages of cerebral β-amyloidogenesis and could furnish clues to the pathogenesis of AD and other diseases involving abnormal protein polymerization.

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