Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8362–8366, October 1991
Medical Sciences

Effects of injected Alzheimer β-amyloid cores in rat brain

(ubiquitin/Alz-50 antigen/lipofuscin)

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Communicated by Roger Guillemin, June 10, 1991

ABSTRACT **Although amyloid deposits have long been known to accumulate in Alzheimer disease (AD) brain, their origin and significance remain speculative. Because of the lack of an *in vivo* model where amyloid deposits can be induced, the relationship of the extracellular β-amyloid deposits to other AD pathology has never been directly investigated. Therefore, we injected SDS-isolated amyloid cores into rat cortex and hippocampus. Similarly isolated lipofuscin fractions from control human brains were injected on the contralateral side. Rats were perfused and brains were examined immunohistochemically at 2 days, 7 days, and 1 month after injection. Alz-50, a monoclonal antibody against abnormally phosphorylated tau proteins, stained neurons along the cortical needle track at 2 but not 7 days after injection of either amyloid or lipofuscin. At 1 month, however, ubiquitin, Alz-50 antigen, and silver-positive structures were observed only in response to amyloid. In 7 of 10 animals, there was considerable neuronal loss in the hippocampal layers. In each instance, these effects were in the immediate vicinity of β-protein immunoreactive material. Marked neuronal loss was never observed at any time after lipofuscin injection. These results indicate a neuronal response to amyloid. When preparations of mature plaque amyloid isolated from the AD brain are injected into the rat brain, they exert neurotoxic effects and induce antigens found in the AD brain.**

Alzheimer disease (AD) is characterized by the presence of neuritic plaques and neurofibrillary tangles, lesions that are accompanied by synaptic and neuronal loss. The core of the plaque consists of a unique 4- to 5-kDa β or A4 peptide (1, 2) and other proteins (3–5). The β protein is a cleavage product of a larger amyloid precursor protein (APP) (6, 7). Deposits in the neuropil are often found compacted in plaque cores surrounded by a halo of dystrophic (8, 9) or sprouting neurites. This configuration suggests that amyloid either derives from the neurites or exerts trophic and/or toxic effects upon them. Ultrastructural studies of AD brain show that amyloid fibrils are often adjacent to profiles of degenerating neurites (10).

Recent studies by several investigators have shown that conditioned medium from cells transfected with cDNA encoding the β protein (and C-terminal regions of APP) and β protein solubilized in acetonitrile are toxic to cultured neurons (8, 11, 12). Further, β protein potentiates the toxicity of glutamate in cultured cortical neurons (13). In contrast to toxicity (12), β protein has also been reported to exert neurotrophic effects (12, 15). Whether insoluble β protein exerts similar effects is unclear. Soluble β protein and related fragments have not been found in AD brain (16). Because insoluble β-protein deposits are abundant in AD brain, we tested the hypothesis that insoluble amyloid cores from AD brain might produce AD-related pathology when injected into rodent brain.

METHODS

Plaque Core and Lipofuscin Preparations. SDS-insoluble amyloid cores were isolated from AD brain (17). Analogous control brain fractions contained principally lipofuscin. The plaque-rich fractions were further purified by sorting on a FACStar^{PLUS} cell sorter (Becton Dickinson) (17).

Plaque core and lipofuscin fractions were washed in phosphate-buffered saline, sterilized with 30, 50, and 70% ethanol, washed twice, and resuspended in sterile saline to provide ≈300 cores (or an equivalent wet weight of the lipofuscin fraction, ≈0.1 μg of protein) in a 3-μl injection volume.

Animals and Surgery. Male and female Sprague–Dawley rats (4–18 months old, 250–390 g, $n = 14$) were anesthetized and placed in a stereotaxic instrument (David Kopf, Tujunga, CA), and β-amyloid cores and control fractions were injected with a 27-gauge Hamilton syringe. The β-amyloid cores were vortex mixed vigorously immediately prior to infusion at 1 μl/2 min into two depths using coordinates from the Paxinos brain atlas (18): in the cortex (1.8 mm ML, −2.80 mm AP Bregma 1.2 DV dura) and in the hippocampus (3.2 mm DV dura). An equal volume and concentration of lipofuscin was injected at the same rate on the contralateral side to provide a matched control. Then 2 days ($n = 2$), 1 week ($n = 2$), and 1 month ($n = 10$) after injection of the β-amyloid core preparation or lipofuscin, the rats were anesthetized and perfused using the pH-shift method; brains were excised and postfixed in a 4% (wt/vol) paraformaldehyde/10% (wt/vol) sucrose solution (19).

Immunohistochemistry. Brains were frozen in dry ice and cryosectioned (20 μm). When sections were examined for Congo red birefringence and autofluorescence, the β-amyloid core and lipofuscin-bearing regions were readily identified. Sections were immunostained for ubiquitin (UBQ) (20) and Alz-50 antigen (8, 24) (P. Davies, Albert Einstein College of Medicine, New York) and counterstained with Congo red (21) or with hematoxylin and eosin. An antiserum [anti-β-(14–24)] raised to a synthetic peptide from β protein [β-(14–24), HQKLVFFAEDVC] (22) was used to detect β-amyloid in rat brains. Immunolabeled sections were processed and developed with diaminobenzidine using the Elite Vectastain kit (Vector Laboratories). For double labeling of amyloid and UBQ, a sequential double-immunoperoxidase procedure was employed (23). Silver staining was performed by the modified Bielschowsky method (9).

RESULTS

Isolation of β-Amyloid Cores and Lipofuscin from Human Brain. As shown in Fig. 1, the anti-β-(14–24) selectively

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Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; UBQ, ubiquitin.

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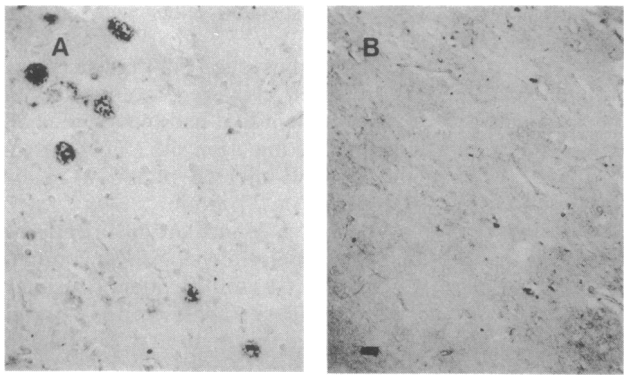

FIG. 1. Immunostaining for β protein in the frontal cortex of a patient with AD. Anti-β-(14–24) labeled amyloid plaque cores in AD brain (*A*), and the labeling was abolished by preincubation with the free β-(14–24) peptide (*B*) (Bar = 10 μm.)

stains amyloid cores in human AD brain (Fig. 1*A*). This staining is blocked by the corresponding peptide at 20 μg/ml (Fig. 1*B*). This serum was used to isolate cores as well as to locate cores after injection. Amyloid cores were isolated from five AD brains and lipofuscin fractions were obtained from two normal human brains in three preparations. SDS-isolated cores stained with anti-β protein were Congo red-positive. As reported (17), the principal contaminant appeared to be lipofuscin, which was not stained by our anti-β-(14–24). The purity of the gradient-isolated preparations was assessed by determining the percentage of Congo red birefringent objects among the total number of objects visible with Congo red, phase-contrast, or autofluorescence. β-Amyloid cores were further purified by cell sorting (≥95% purity) (Fig. 2*A*). Many of the birefringent profiles showed the typical maltese-cross configuration (Fig. 2 *B–D*) and were immunopositive for anti-β-(14–24). SDS-isolated cores have been shown to contain α_1-antichymotrypsin and heparan sulfate proteoglycan.

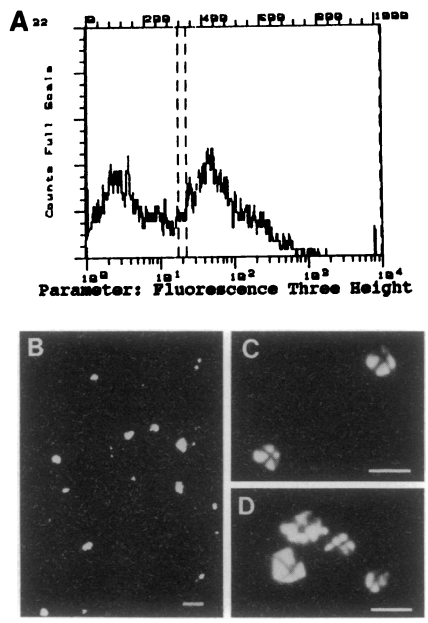

FIG. 2. Purification of plaque cores from human brains. SDS-gradient-isolated plaque cores from five brains were compared for purity by light microscopic examination and selected preparations were used for additional purification on a FACStar cell sorter. (*A*) A typical cell-sorter trace for the first autofluorescent sort. The populations of bright and dull particles were sorted as indicated and examined for Congo red birefringence and β-protein staining. The less fluorescent peak on the left was used for the plaque core preparation. (*B–D*) Congo red-positive cores of a typical preparation. (Bar = 10 μm.)

Fate of β-Amyloid and Lipofuscin 1 Month After Injection. Fig. 3 shows the typical dispersion of injected material in the cortex and hippocampus 1 month after injection. In the cortex, lipofuscin (Fig. 3*A*) and amyloid (Fig. 3*B*) did not diffuse away from the needle wound but persisted in the vicinity of the needletrack. Hematoxylin and eosin staining confirmed the location of the cortical needle tracks. However, in the hippocampus, lipofuscin (Fig. 3*C*) and amyloid (Fig. 3*D*) typically migrated posteriorly and medially from the injection site, just ventral and parallel to the hippocampal fissure. Autofluorescence (Fig. 3*E*) and Congo red birefringence (Fig. 3*F*) confirmed the dispersion in the hippocampus of the lipofuscin and β-amyloid cores, respectively. We verified the fate of β-amyloid after injection by immunostaining.

Immunoreactivity to Alz-50 and UBQ After Injection of β-Amyloid. Because UBQ is an excellent marker for early neuritic degeneration either associated with plaques (9) or induced after glutamate toxicity in the rat (24), we examined UBQ in rat brains injected with β-amyloid. At 2 days, there was an increase in UBQ in the vicinity of the needletracks of both amyloid- or lipofuscin-injected sites (data not shown). At 1 week, there was some UBQ remaining in the lipofuscin-injected site, but there were considerably more profiles immunostained for UBQ surrounding β protein, determined by immunoreactivity or Congo red birefringence. After 1 month, in all 10 animals, UBQ persisted around the amyloid but was not associated with lipofuscin (Fig. 4). Along the needletrack in the cortex where birefringence was observed, staining for UBQ showed a few linear profiles and several macrophages (Fig. 4*A*). On an adjacent section, blocking with UBQ (10 μg/ml) prevented the staining (Fig. 4*B*), and no UBQ was apparent on the contralateral needletrack in the

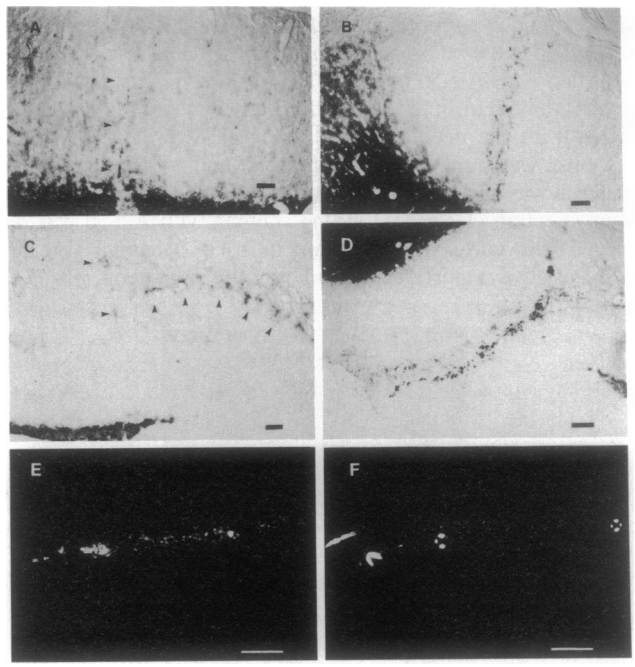

FIG. 3. Distribution of β-amyloid cores and lipofuscin in the rat cortex and hippocampus 1 month after injection. (*A* and *B*) Modified Bielschowsky silver staining of the needle tracks on the control and amyloid sides in the cortex. (*C* and *D*) Injected cores spread medially across the hippocampus. Arrowheads demarcate the needletrack and distribution of lipofuscin on the contralateral control side. (*E*) Autofluorescent lipofuscin distributed across the hippocampal fissure on the control side. (*F*) Congo red birefringence of the injected amyloid. (Bar = 100 μm.)

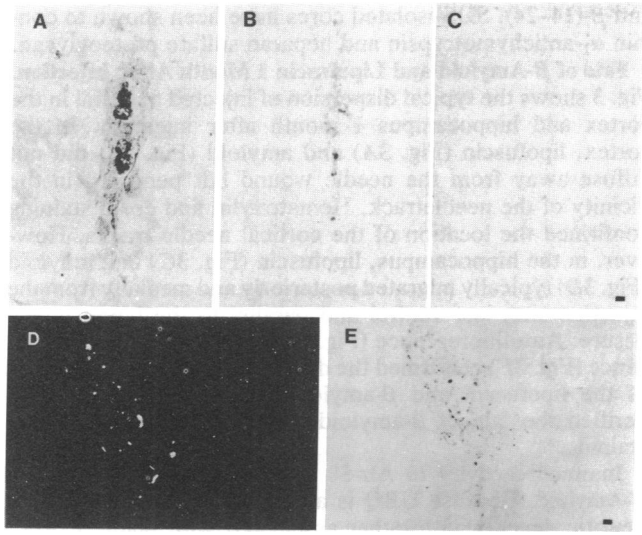

FIG. 4. UBQ immunoreactivity in the rat brain 1 month after injection of β-amyloid cores. (*A*) UBQ in the cortex on the amyloid side at 1 month. (*B*) Incubation of sections with anti-UBQ preabsorbed with UBQ dramatically reduced staining although there were several phagocytes with yellow pigment. (*C*) The contralateral side in the cortex revealed negligible UBQ. (*D*) Congo red birefringence on the amyloid-injected side parallel to the hippocampal fissure where the amyloid appears to enter the ventricle. (*E*) The same section stained for UBQ. (Bar = 10 μm.)

vicinity of the lipofuscin (Fig. 4*C*). In the hippocampus, distal to the needletrack, staining for UBQ revealed several punctate profiles (Fig. 4*E*) that closely paralleled the pattern of birefringence (Fig. 4*D*).

In AD brain, dystrophic neurites that stain with Alz-50 are often observed surrounding β-amyloid cores (8). Therefore, we also examined sections stained with Alz-50 after injection of β-amyloid in the rat brain. Although our amyloid preparation did not stain with Alz-50 or anti-UBQ, at 2 days after injection of either amyloid or lipofuscin, Alz-50-reactive material was associated with the needletrack. By 1 week, however, the initial response was diminished at both sites, and by 1 month Alz-50 material was absent on the control side. In contrast, at 1 month after injection of amyloid strong staining with Alz-50 was observed in the cortex and hippocampus in 4 of 10 animals (Fig. 5*B*) that was always clustered around birefringent material (Fig. 5*A*).

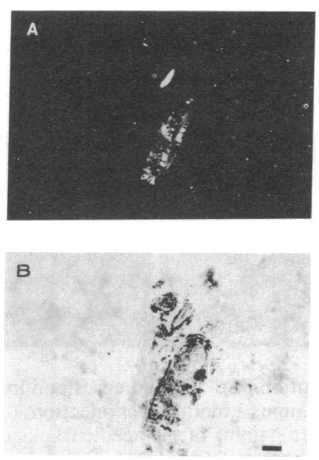

FIG. 5. Immunostaining with Alz-50 in the rat brain 1 month after injection of β-amyloid cores. Congo red birefringence in the hippocampus (*A*) and immunostaining with Alz-50 (*B*) in the same section. (Bar = 10 μm.)

Alz-50-reactive material was associated either with cell processes or phagocytes.

Double immunolabeling studies showed that structures containing UBQ could be seen clustered around amyloid cores immunostained for β protein that had moved medially in the hippocampus far from the injection site (Fig. 6). Some phagocytic cells in the amyloid-injected region were also observed to contain UBQ and/or β protein.

Bielschowsky Silver Staining 1 Month After Injection of β-Amyloid in the Rat Brain. Bielschowsky silver staining, although not entirely specific, is useful for identification of dystrophic neurites, neurofibrillary tangles, and β-protein deposits in AD brain (9). We used this method to examine the effects of injected β-amyloid in the rat brain. At 2 and 7 days after injection of purified cores, except for small glial cells and myelinated nerve fibers, only the injected cores were silver-positive, with our development times (data not shown). However, at 1 month after injection, several silver-positive profiles were observed that were not present on the lipofuscin-injected side (Fig. 7). There was intense silver impregnation (Fig. 7*A*) in the vicinity of Congo red birefringence (see Fig. 3*F*). In contrast, the contralateral side injected with lipofuscin showed only light silver impregnation of small glial cells (Fig. 7*B*) in the region of autofluorescence (see Fig. 3*E*). Higher magnification of the amyloid-injected side, but not the control side, revealed many silver-positive linear and punctate profiles, tentatively identified as dystrophic neurites, that were frequently associated with the silver-impregnated processes (Fig. 7 *C* and *D*).

Neuron Loss After Injection of β-Amyloid in the Rat Hippocampus. In all animals, there was no neuron loss in the hippocampus at 2 days and at 7 days after injection of amyloid (data not shown). By 1 month, however, the loss in the hippocampus was dramatic (Fig. 8). In the hippocampus, β-amyloid cores and lipofuscin migrated to slightly different locations from animal to animal. When the amyloid was concentrated adjacent to neuronal perikarya (seven animals), a marked neuronal loss was obvious by visual inspection, particularly in the dentate gyrus (Fig. 8 *B* and *D*). In contrast, this was never observed in the lipofuscin-injected side (10 animals, Fig. 8 *A* and *C*). Reactive gliosis occurred on both the lipofuscin- and amyloid-injected sides but was much more marked in the hippocampus on the amyloid-injected sides in regions of neuron loss.

The neuron loss in the hippocampus 1 month after injection of lipofuscin or amyloid is quantified in Table 1. In the three animals where the β-amyloid cores injected into the hippocampus did not become positioned near neuron layers, there was no neuron loss observed. In the four animals where the injection of β-amyloid resulted in its medio-lateral distribution along the layers of the

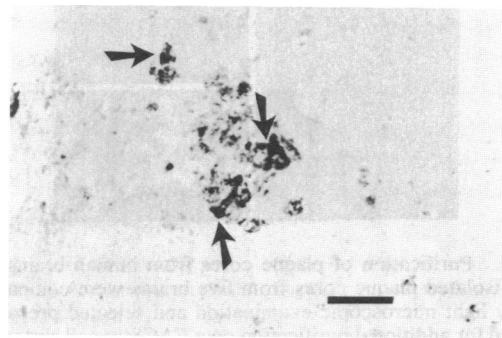

FIG. 6. Double labeling for β protein and UBQ in the rat hippocampus 1 month after medial injection of β-amyloid cores. Sections were immunostained for β protein with nickel chloride enhancement (black, shown by arrows) followed by dehydration and labeling for UBQ with diaminobenzidine alone (gray). (Bar = 10 μm.)

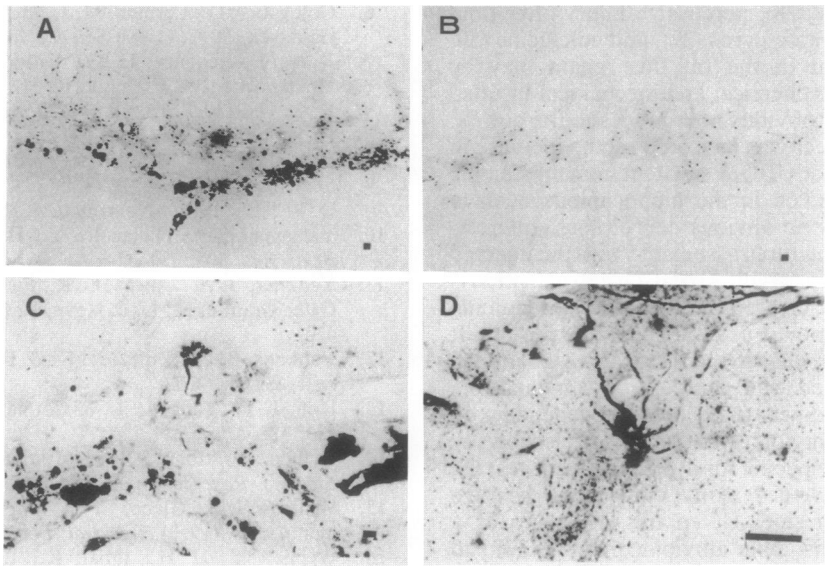

FIG. 7. Modified Bielschowsky silver stains of rat brain sections 1 month after injection of β-amyloid. Several silver-positive profiles were associated with the injected amyloid suggestive of dystrophic neurites (*A*, *C*, and *D*). Similar profiles were rare on the lipofuscin-injected side (*B*). (Bar = 10 μm.)

dentate gyrus, the neuron loss was dramatic in the outer or inner molecular layer of the dentate gyrus associated with the amyloid. Similar dispersion of the control lipofuscin did not result in cell loss. In two additional amyloid-injected animals, most of the cores remained along the needle track entering the hippocampus in the CA1 pyramidal layer of the hippocampus where significant neuron loss was observed. In one animal, most of the β-amyloid became distributed between the inner and outer molecular layer of the dentate gyrus; this animal had considerable neuron loss in the adjacent medial CA3 pyramidal layer of the hippocampus. Amyloid-associated neurotoxicity was observed in aged and young animals and in female and male animals. Additional controls with injected agarose beads (Pharmacia) also failed to show neuron loss. Although injection of amyloid into the cerebral cortex caused increased staining with Alz-50 and for UBQ by 1 month, it did not cause obvious cortical neuron loss.

DISCUSSION

SDS-insoluble amyloid fractions from AD brain can produce neurotoxic effects when injected into the rat brain. The most dramatic evidence for neurotoxicity is hippocampal neuron loss in the immediate vicinity of the amyloid. This was evident in 7 of 10 animals at 1 month. Neither lipofuscin nor agarose beads injected with the same coordinates, rate, and volume on the contralateral side produced this toxicity, despite the microglia/macrophages and lipofuscin autofluorescent tracks involving the same neuronal layers. Lipofuscin injection on the contralateral side is an ideal control because it simultaneously reflects the potential effects of injection, of the vehicle, of unknown contaminants present in normal brain, of trace detergent, and of insoluble foreign human protein persisting in the preparation. Neuron loss was especially dramatic in four animals where the amyloid spread parallel to the dentate gyrus. This may reflect intrinsic vulnerability of this region or a tendency of injected cores to spread readily in the dentate layer. In support of the hypothesis that the dentate gyrus may have an affinity for the amyloid, the distribution of the injected cores in the rat is surprisingly similar to the linear orientation of plaques in the inner third molecular layer of the dentate gyrus in AD (25–27). The amyloid-associated neuron loss is similar to that

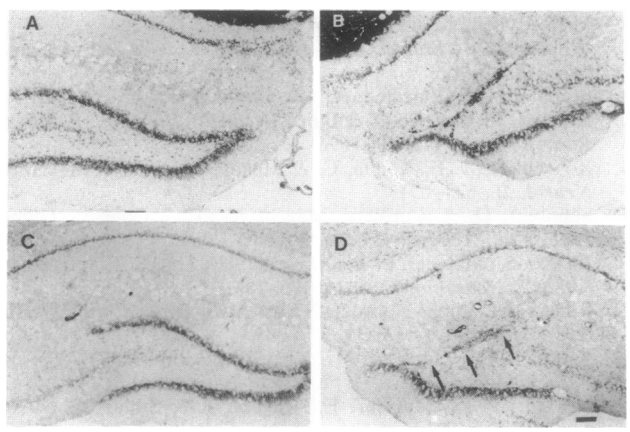

FIG. 8. Neuron loss in the rat dentate gyrus 1 month after injection of β-amyloid. Hematoxylin and eosin-stained sections revealed a marked neuronal cell loss in the vicinity of the injected amyloid but not lipofuscin. (*A* and *B*) Modified Bielschowsky silver stains. (*C* and *D*) Immunostaining with anti-β-(14–24). (*A* and *C*) Control side. (*B* and *D*) Amyloid side. Arrows show cell loss. (Bar = 100 μm.)

Table 1. Effect of β-amyloid plaque cores or lipofuscin on hippocampal neuron loss 1 month after injection

Rat	Plaque-associated cell loss	Region with plaque-associated cell loss	Cell loss (AP × ML)
Alz 1	+++	Outer DG	0.80 × 1.50
Alz 2	+++	Outer DG	0.72 × 1.70
Alz 3	+	CA1	0.25 × 0.25
Alz 4	+	CA1	0.25 × 0.25
Alz 5	−	N/A	N/A
Alz 9	−	N/A	N/A
Alz 11	+++	Inner DG	0.65 × 2.00
Alz 13	++	Medial CA3	0.50 × 0.75
Alz 15	−	N/A	N/A
Alz 16	+++	Outer DG	0.75 × 1.8

Degree of cell loss is indicated by +++ (dramatic), ++ (considerable), + (moderate), or − (negligible). DG, dentate gyrus. The dimensions of the anterior–posterior (AP) and medio-lateral (ML) cell loss are indicated in mm (AP × ML) and were spatially correlated with the distribution of the plaque cores. There was no lipofuscin-associated cell loss (<0.1 mm) observed. NA, not available.

seen after adrenalectomy (28), percussive injury after fluid injection directly into dentate gyrus (29), and colchicine (30, 31), which suggests that in the rat this region may be particularly vulnerable to chemical and mechanical insults.

Amyloid did not cause obvious neuron loss in the cortex. Nevertheless, a toxic effect was indicated by the increase in staining with Alz-50 and for UBQ. Persistent immunostaining for UBQ was also observed in the hippocampus even in animals where there was no obvious neuron loss. Interestingly, some but not all structures associated with the injected amyloid were immunostained with Alz-50. One explanation is that these structures were cleared since we also saw staining with Alz-50 in some phagocytic cells adjacent to β protein. It is important to note that similar to the AD brain (8, 20) not all of the cores are associated with neurotoxicity. Indeed, the acute neurotoxic effects appear to be local and dependent on the exact location of the amyloid. Although the Bielschowsky silver stain is not entirely specific for dystrophic neurites, the amyloid-injected side revealed many more silver-positive profiles than the control side. Given this evidence for a specific toxic effect of insoluble amyloid, many additional experiments are now necessary to elucidate the mechanism of neurotoxicity.

In vitro experiments with cultured neuronal cell lines and rat primary neuronal cultures have shown that a fragment of APP generated from a transfected cDNA that includes most of the β protein and the C terminus of APP is toxic to neurons (11), and β-(1–42) has been found to potentiate excitotoxicity in cultured mouse neurons (13). Further, the synthetic peptides of β protein, β-(1–28) or β-(1–42), appear to have trophic and neurite-promoting activity (12, 15, 32). Because Yankner *et al.* (12) have also reported trophic and toxic effects at low doses of β-(1–40) *in vitro*, even the activity of this acetonitrile-solubilized peptide appears highly variable and it is unclear what levels exist *in vivo*. We have sought to determine the *in vivo* effects of AD plaque cores themselves. Because amyloid cores may serve to sequester factors such as basic fibroblast growth factor (33), protease nexin I (34), and other heparin-binding proteins, like APP (35), the effects of the cores may not be direct. Finally, amyloid plaques in AD brain appear to provoke a vigorous microglial inflammatory response, which in itself may produce toxic effects (36–38). It is thus interesting to note that antiinflammatory therapy is correlated with a low prevalence of AD (39).

We thank Drs. R. Terry and E. Masliah for AD brains and valuable advice, Ms. Judy Nordberg (Veterans Hospital, La Jolla) for flow cytometry sorting, Anne Fagan for helpful suggestions, and Ali Vazirizand for technical assistance. This work was supported by National Institutes of Health Grants DK-18811 (A.B.), NS-28121 (A.B.), and AG-09009 (G.M.C.) and the Department of Health Services, State of California (G.M.C.).

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