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β-Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red

(Alzheimer disease/synapse loss/amylin/diabetes)

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ABSTRACT **β-Amyloid (βA) is normally produced as a nontoxic soluble peptide. In Alzheimer disease, βA aggregates and accumulates in the brain as inert diffuse plaques or compact plaques associated with neurodegenerative changes. To determine the relationship of neurotoxicity to the physical state of βA, we created (*i*) nonamyloidogenic amorphous aggregates of βA [amorphous βA (Am-βA)] analogous to diffuse plaques and (*ii*) amyloidogenic fibrils of βA [fibrillar βA (Fib-βA)] analogous to compact plaques. In primary rat hippocampal culture, Fib-βA was neurotoxic, whereas Am-βA was not toxic. Fib-βA caused significant loss of synapses in viable neurons, while Am-βA had no effect on synapse number. The amyloid fibril-binding dye Congo red inhibited Fib-βA neurotoxicity by inhibiting fibril formation or by binding to preformed fibrils. Congo red also inhibited the pancreatic islet cell toxicity of diabetes-associated amylin, another type of amyloid fibril. These results indicate that βA neurotoxicity requires fibril formation. These findings and our previous demonstration that amylin fibrils are toxic suggest that a common cytopathic effect of amyloid fibrils may contribute to the pathogenesis of Alzheimer disease and other amyloidoses.**

The formation of insoluble deposits of β-amyloid (βA) in the brain is a pathological hallmark of Alzheimer disease. The βA peptide is normally produced as a soluble metabolic product of the amyloid precursor protein (1–7). In Alzheimer disease, βA aggregates and accumulates in the brain as diffuse and compact plaques. Diffuse plaques are not associated with degenerative changes, whereas compact plaques composed of βA fibrils are associated with pathological changes in the surrounding brain parenchyma (reviewed in ref. 8). The basis of this differential neuronal response is unclear but may relate to the different physical states of βA in diffuse and compact plaques.

The βA peptide is neurotoxic in cell culture and *in vivo* (9–12). Aggregation of βA appears to be necessary to achieve a toxic state (13–17), although the precise physical form of the peptide that mediates toxicity is unknown. We recently demonstrated that diabetes-associated amylin is toxic to insulin-producing islet cells of the pancreas and showed that the toxic activity is mediated by the fibrillar form of the peptide (18). In this report, we have determined that βA neurotoxicity is mediated by the βA fibril, suggesting that a common mechanism of cell death may be operative in many diseases associated with amyloid fibril formation. Congo red inhibited the cytotoxicity of βA and amylin fibrils, suggesting a potential therapeutic approach to Alzheimer disease and other amyloidoses.

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MATERIALS AND METHODS

Peptides. Synthetic βA and amylin-(1–37) peptides were obtained from Bachem; βA in senile plaques comprises 40- to 43-amino acid polypeptides of which the 42-residue protein is the predominant form (31). Amorphous aggregates of βA-(1–42) (Am-βA) were produced by dissolving the lyophilized peptide in phosphate-buffered saline (pH 7.3) to 350 μM. Aggregates formed immediately and were collected by centrifugation at 5600 × *g* for 1 min, resuspended in phosphate-buffered saline (PBS), and added to cultures immediately. Fibrils of βA-(1–42) (Fib-βA) were produced by dissolving the peptide in double-distilled water (ddH_2O) to 350 μM and incubating at 37°C for 3 days. Fibrils of β-(1–40) were produced by dissolving the peptide in ddH_2O to 700 μM, immediately diluting in PBS to 350 μM and incubating at 37°C for 5 days. The βA solutions were diluted to 20 μM in hippocampal cultures. Aggregates of Am-βA-(1–42), Fib-βA-(1–42), and Fib-βA-(1–40) were stable in culture medium, whereas Am-βA-(1–40) dissolved gradually. Therefore, Am-βA-(1–40) was not used for comparison with fibrils. Amylin was dissolved in ddH_2O to 350 μM and diluted immediately into islet cell cultures to 8 μM. Electron microscopy and Congo red staining were performed as described (18).

Cell Culture. Primary rat hippocampal cultures were prepared as described (9) and plated at 175 cells per mm^2 in 16-mm poly(L-lysine)-coated wells. After 4 days in culture, the medium was changed to serum-free Dulbecco's modified Eagle's medium with N2 and B27 supplements (GIBCO). One day later, peptides were added for a 3-day incubation. Neuronal viability was determined as described (9) in five 0.4-mm^2 fields per well in triplicate wells (>300 neurons scored in controls). Rat pancreatic islet cell cultures were prepared, and cell viability was determined by propidium iodide staining as described (18). For immunocytochemistry, cultures were fixed in PBS containing 4% paraformaldehyde and 0.12 M sucrose, blocked with 5% (vol/vol) bovine serum albumin, and then incubated with primary antibody for 12 hr at 4°C [microtubule-associated protein 2 (MAP-2) monoclonal antibody AP-20 from Sigma, 1:500; synaptophysin monoclonal antibody from Boehringer Mannheim, 1:200]. Cells were incubated with biotinylated anti-mouse IgG (1:200) and developed by using the ABC kit (Vector Laboratories) and diaminobenzidine. Presynaptic terminals were scored in neurons maintained for 8 days in culture and immunocytochemically stained for synaptophysin by counting synaptophysin dots in the initial 30 μm of a dendrite in 30 identified viable neurons (>450 synapses scored in controls).

Abbreviations: βA, β-amyloid; Fib-βA fibrillar βA; Am-βA amorphous βA; MAP-2, microtubule-associated protein 2.

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RESULTS

To test the hypothesis that the differential neurodegenerative changes surrounding immature diffuse plaques and mature compact plaques reflect differences in the physical state of βA, we created *in vitro* analogs of βA in the two plaque types. Dissolution of βA-(1–42) directly into PBS resulted in rapid aggregation, giving rise to amorphous aggregates, as determined by electron microscopy (Fig. 1*A*). Dissolution of βA-(1–42) in distilled water and incubation at 37°C for 3 days resulted in a pure preparation of 6- to 9-nm βA fibrils (Fig. 1*B*). Congo red staining and examination under polarized light demonstrated intense birefringence of the βA fibrils, similar to that of βA in compact plaques in the brain (Fig. 1*D*). The amorphous aggregated βA was not birefringent with Congo red stain, similar to the βA in diffuse plaques (Fig. 1*C*).

The biological effects of Am-βA and Fib-βA were determined in primary rat hippocampal cultures (9). Immunocytochemical staining of neurons for MAP-2 showed that Fib-βA induced neurodegenerative changes including dystrophic neurites, shrinkage of the soma, and neuronal loss (Fig. 2 *A* and *C*). Neuronal cultures treated with Am-βA were morphologically indistinguishable from controls (Fig. 2 *A* and *B*). Quantitation of neuronal cell number demonstrated that Fib-βA caused significant neuronal cell loss, whereas Am-βA was not toxic (Table 1). Prolonged incubation of Am-βA for 7 days *in vitro* resulted in the conversion of some of the peptide to a Congo red birefringent form; addition of this peptide to hippocampal cultures resulted in neurotoxicity (data not shown).

Synapse loss is an early pathological change in Alzheimer disease that correlates with the degree of cognitive impairment (19). To determine if βA can cause synapse loss, we examined rat hippocampal neurons grown in culture for 8 days. Immunocytochemical staining of synaptophysin showed a dense coating of punctate synaptophysin immunoreactivity along the dendrites, characteristic of presynaptic terminals (Fig. 2*D*). Treatment with Fib-βA resulted in a marked reduction in the density of presynaptic terminals along dendrites (Fig. 2*F*). Quantitation of synapse number demonstrated that Fib-βA caused a 60 ± 6% loss of presynaptic terminals (mean ± SEM, $P < 0.001$) (Table 1). Double-labeling with the vital stain propidium iodide demonstrated that synapse loss occurred in neurons still viable after treatment with Fib-βA (data not shown), suggesting that synapse loss precedes neuronal death. Treatment with Am-βA did not significantly affect the number of presynaptic terminals (Fig. 2*E* and Table 1). These results suggest that βA-induced

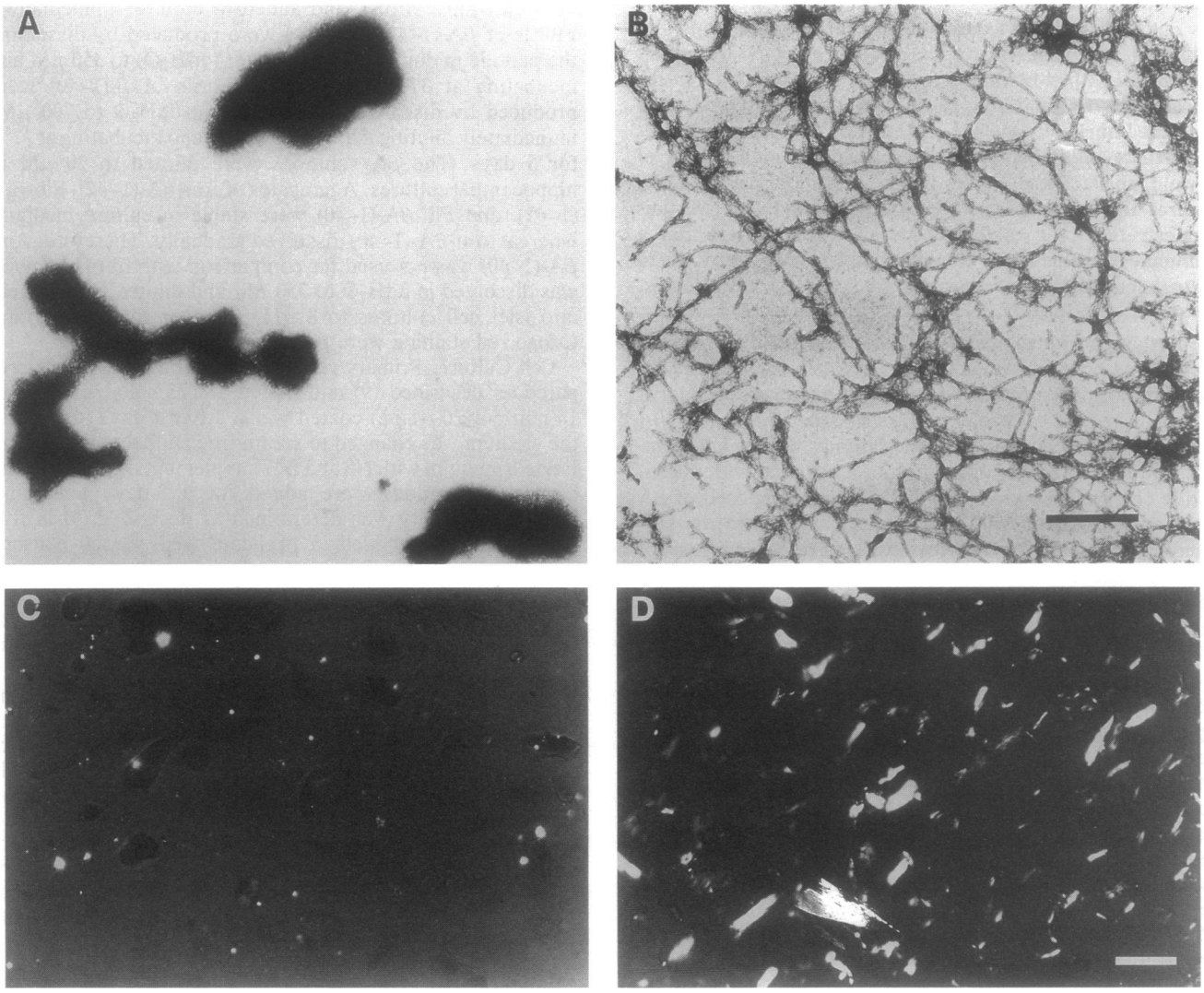

FIG. 1. Amorphous and fibrillar preparations of βA. Preparations of βA were analyzed by electron microscopy (*A* and *B*) and Congo red staining followed by illumination with polarized light (*C* and *D*). Rapid aggregation of the βA-(1–42) peptide in PBS produces amorphous aggregates that are not birefringent with Congo red stain (*A* and *C*). Incubation of βA-(1–42) for 3 days in water produces 6- to 9-nm βA fibrils that are intensely birefringent with Congo red stain (*B* and *D*). (Bars = 200 nm in *B* and 50 μm in *D*.)

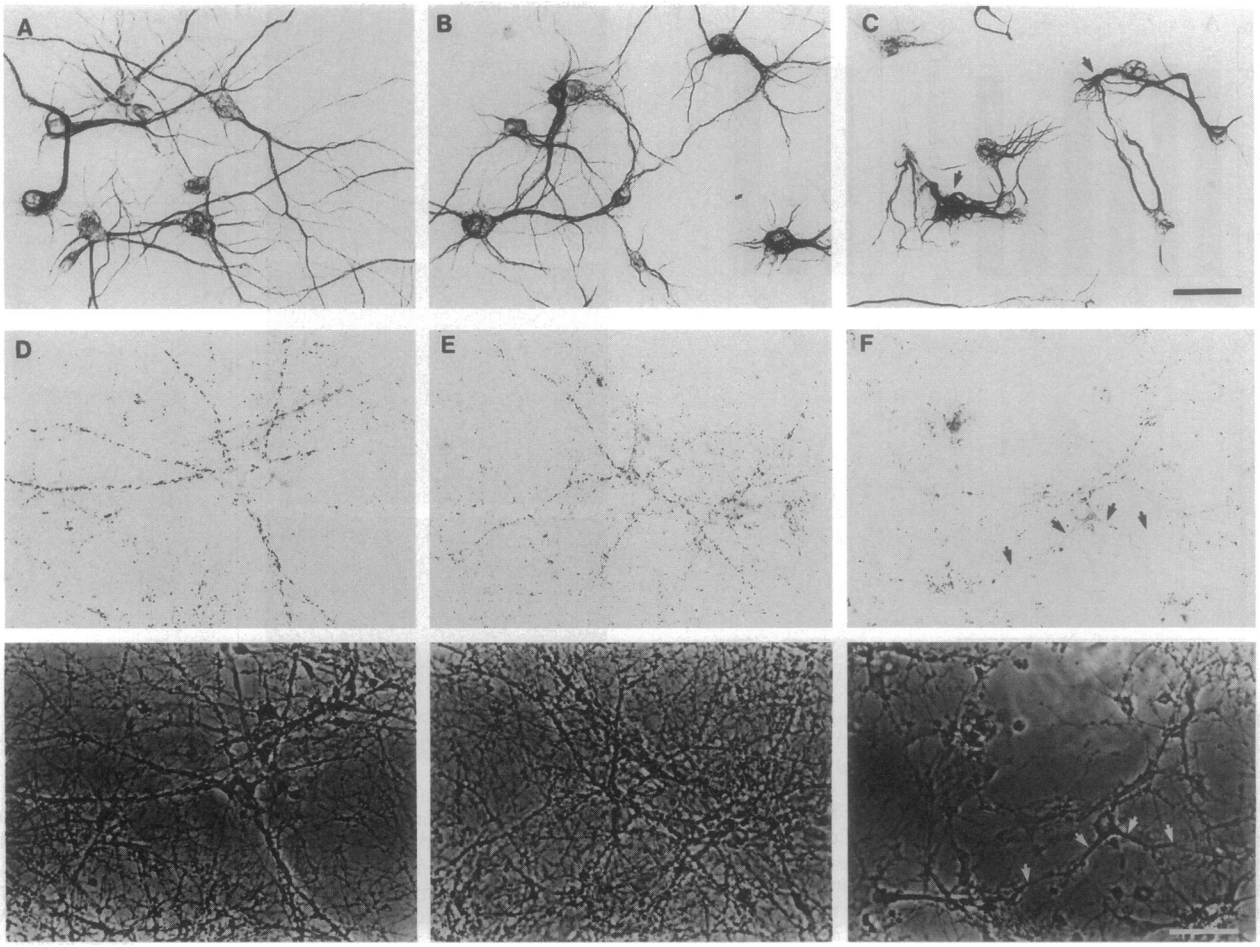

FIG. 2. Neurodegenerative effects of the βA fibril. Hippocampal cultures were immunocytochemically stained for MAP-2 (*A–C*) or synaptophysin (*D–F*; *Upper*, bright field; *Lower*, phase contrast). (*A* and *D*) Control. (*B* and *E*) Am-βA. (*C* and *F*) Fib-βA. Note that Fib-βA causes neurite retraction and dystrophic changes (arrows in *C*) and a marked decrease in the density of presynaptic terminals along dendrites (arrows in *F*). Neurons treated with Am-βA (*B* and *E*) are indistinguishable from controls (*A* and *D*). Hippocampal cultures were incubated with 20 μM of βA peptides for 3 days. (Bars = 40 μm).

neurotoxicity and synapse loss are mediated by the fibrillar form of the peptide.

To confirm that amyloid fibrils mediate βA toxicity, we examined the effect of the amyloid fibril-binding dye Congo red. Preincubation of soluble βA-(1–40) with an equimolar concentration of Congo red followed by addition to the culture medium resulted in complete inhibition of neurotoxicity (Fig. 3*A*). Maximal neuroprotection was observed at a molar ratio of 0.35:1 (Congo red/βA) when Congo red was added to soluble peptide prior to preincubation (Fig. 3*C*). The effect of Congo red on fibril formation was determined by separation of the soluble and insoluble peptide by centrifugation. Electron microscopy confirmed that fibrils were selectively recovered in the insoluble fraction contained in the pellet. Immunoblot (Western blot) analysis demonstrated that preincubation of βA-(1–40) with Congo red significantly reduced the level of insoluble peptide and increased the level of soluble peptide (Fig. 3*D*), indicating that Congo red inhibited fibril formation.

Table 1. Effects of Am-βA and Fib-βA on neuronal viability and synapse number

Treatment	Viable neurons, %	Presynaptic terminals, %
Control	100 ± 4	100 ± 7
Am-βA	91 ± 4	85 ± 5
Fib-βA	53 ± 3*	40 ± 6*

Hippocampal cultures were incubated with 20 μM of Am-βA- or Fib-βA-(1–42) for 3 days, and neuronal viability was determined. The number of synaptophysin-immunoreactive presynaptic terminals was determined in the remaining viable neurons as described in *Materials and Methods*. Values are expressed as percent of control and represent the mean ± SEM (n = 15 for cell viability; n = 30 for presynaptic terminals) in a representative experiment.
*, $P < 0.001$ relative to control by ANOVA with post hoc Tukey test.

A second mechanism of neuroprotection was evident when Congo red was added to preformed βA-(1–40) fibrils. Fibrils were preformed *in vitro* and then incubated with an equimolar concentration of Congo red for 24 hr. The fibrils were then separated from unbound Congo red by centrifugation. The separated fibrils were birefringent under polarized light (as in Fig. 1*D*), indicating that they were bound to Congo red. These fibrils were not toxic (Fig. 3*B*), indicating that Congo red binding to βA fibrils completely inhibits their neurotoxicity. Congo red also inhibited the neurotoxicity of βA-(1–42) by binding to fibrils (Fig. 3*A*) but did not inhibit fibril formation (data not shown). Thus, Congo red can protect against βA neurotoxicity either by inhibiting fibril formation or by binding to preformed fibrils.

We have demonstrated (18) that the fibrillar form of amylin that accumulates in the pancreas in type 2 diabetes mellitus is toxic to insulin-producing islet cells. The effect of Congo red on the toxicity of amylin fibrils was examined in primary cultures of rat pancreatic islet cells. Addition of amylin resulted in marked degenerative changes and 62 ± 3% islet cell death after 24 hr (Fig. 4 *A–D*). Addition of Congo red to

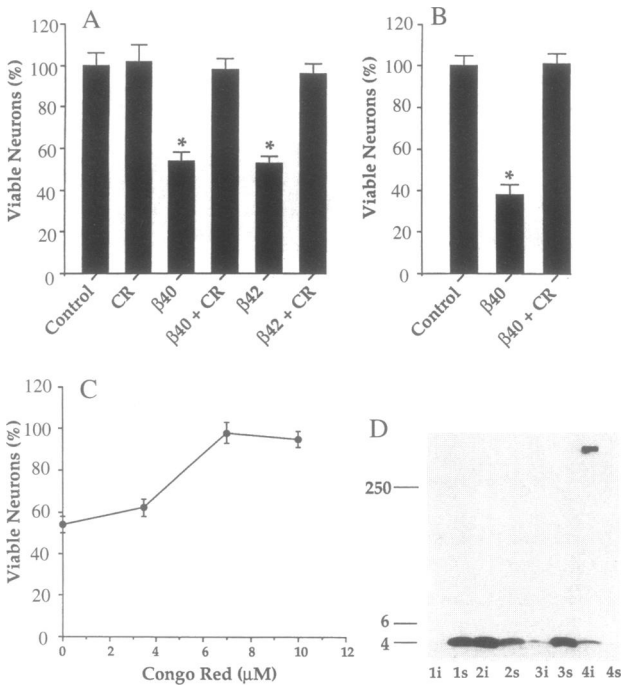

FIG. 3. Inhibition of βA neurotoxicity by Congo red. (*A*) Preincubation of βA-(1–40) (β40) or βA-(1–42) (β42) with Congo red inhibits neurotoxicity. Soluble βA peptides (350 μM) were preincubated alone or with Congo red [350 μM with βA-(1–40); 700 μM with βA-(1–42)] as described in text and then added to hippocampal cultures [final βA concentration, 20 μM, final Congo red concentrations, 20 μM with βA-(1–40) and 40 μM with βA-(1–42)]. Neuronal viability was determined after 3 days. (*B*) Congo red binds to preformed βA fibrils and inhibits neurotoxicity. Fibrils of 350 μM βA-(1–40) preformed *in vitro* were incubated with 350 μM Congo red for 24 hr. Fibrils were separated from free Congo red by centrifugation at 13,000 × *g* for 5 min and added to hippocampal cultures (final βA concentration, 20 μM). Values are expressed as percent of control and represent the mean ± SEM ($n = 15$). *, $P < 0.001$ relative to control by ANOVA with post hoc Tukey test. (*C*) Concentration dependence of Congo red inhibition of βA neurotoxicity. Preincubation of βA-(1–40) (350 μM) with Congo red (0–175 μM) was performed as described in *A* followed by addition to hippocampal cultures to a final βA concentration of 20 μM and the indicated concentrations of Congo red. (*D*) Preincubation of Congo red with βA-(1–40) inhibits fibril formation. Lanes: 1, 20 μM βA-(1–40) immediately after solubilization; 2, βA-(1–40) preincubated at 37°C for 5 days; 3, βA-(1–40) preincubated with Congo red as described in *A*; 4, Congo red added to preformed βA-(1–40) fibrils as described in *B*. After incubation, the peptide solutions were centrifuged at 13,000 × *g* for 5 min, and equivalent aliquots of insoluble βA in the pellet (lanes i) and soluble βA in the supernatant (lanes s) were resolved by Tris-tricine SDS/10–20% PAGE, Western blotted with the βA-(1–40) polyclonal antibody B6 (7), and developed by enhanced chemiluminescence (Amersham). Note that preincubation with Congo red markedly decreases the level of insoluble βA and increases the level of soluble βA (lanes 3i and 3s compared with lanes 2i and 2s).

islet cell cultures completely inhibited amylin toxicity (Fig. 4 *C* and *D*). Centrifugation of the peptide after incubation for 24 hr showed that the peptide had converted entirely to the insoluble form that is composed of amylin fibrils (Fig. 4*E*) (18). Congo red did not inhibit amylin fibril formation. As observed for βA, amylin fibrils incubated with Congo red became birefringent, indicating that Congo red binding to fibrils renders them nontoxic. These results suggest that Congo red is a general inhibitor of amyloid fibril toxicity.

DISCUSSION

These experiments demonstrate that the neurotoxicity of βA is mediated by the amyloid fibril. Amorphous aggregated βA

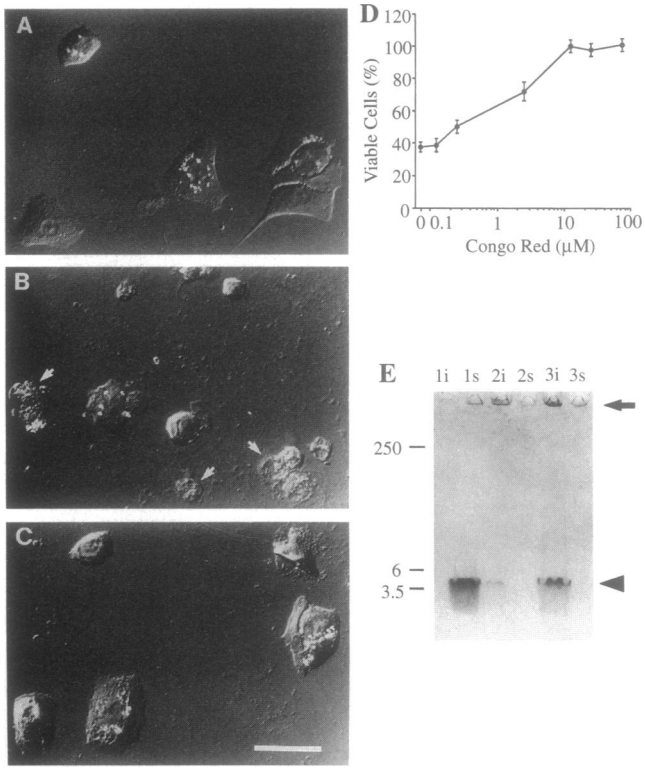

FIG. 4. Pancreatic islet cell toxicity of amylin is inhibited by Congo red. (*A*) Primary rat pancreatic islet cells. (*B*) Degenerative changes (arrows) after incubation with 8 μM human amylin for 24 hr. (*C*) Protection against islet cell degeneration by addition of 12.5 μM Congo red with amylin. (Bar = 40 μm). (*D*) Concentration dependence of Congo red inhibition of amylin toxicity. Islet cells were incubated for 24 hr with 8 μM amylin in the absence or presence of the indicated concentrations of Congo red. Values are expressed as percent of control and represent the means ± SEM ($n = 15$). Congo red concentrations of 2.5 μM or greater provided significant islet cell protection ($P < 0.001$). (*E*) Congo red does not prevent amylin fibril formation. Amylin was preincubated in PBS for 24 hr in the absence or presence of Congo red and then centrifuged to separate the fibrillar insoluble peptide (lanes i) from soluble peptide (lanes s) as described (18). Lanes: 1, freshly solubilized amylin is recovered as a soluble 4-kDa peptide; 2, preincubated amylin is recovered in the insoluble fraction as a 4-kDa peptide (arrowhead) and as large aggregates that do not enter the gel (arrow); 3, amylin preincubated with Congo red is recovered entirely in the insoluble fraction. Peptide fractions were resolved by Tris glycine SDS/4–20% PAGE and stained with Coomassie brilliant blue. Sizes are shown in kDa.

is not toxic. This observation establishes a correlation between the cell culture model of βA neurotoxicity and the pathology of Alzheimer disease. In the Alzheimer brain, βA deposits form in two basic types of plaques, immature diffuse plaques and mature compact plaques. Diffuse plaques are not associated with neurodegenerative changes, are Congo red-negative, and contain Am-βA aggregates and fibrils (20–22). In contrast, compact plaques are associated with neurodegenerative changes, are Congo red-positive, and contain a high density of βA fibrils. Our results suggest that the neurotoxicity associated with compact plaques may be the result of the high density of βA fibrils.

We have demonstrated that βA fibrils cause synapse loss prior to neuronal cell death. This may be due to a direct effect of the βA fibril at the synapse or may be secondary to βA-induced axonal retraction. Synapse loss is an early pathological change in the Alzheimer brain that correlates with the severity of dementia (19, 23). Synapse loss is associated with amyloid plaques but is also observed in brain regions devoid of plaques (19). Our results raise the possibility that synapse

loss could occur at an early stage of βA fibril deposition, resulting in neuronal disconnection prior to neuronal degeneration.

Several studies suggest that the neurotoxicity of βA correlates with the formation of βA aggregates and that variation in the neurotoxic potency of different synthetic preparations of βA relate to differences in peptide aggregation (13–17). Neurotoxicity has also been correlated with the degree of β-sheet structure of βA in solution (24). However, we have previously reported that other peptides, such as glucagon, that posses β-sheet structure but remain soluble in solution are not toxic (9). Therefore, it is likely that acquisition of β-sheet structure correlates with βA toxicity because it is a prerequisite for fibril formation, since β-sheet structure is not by itself sufficient to cause neurotoxicity. Furthermore, the toxic potency of an amyloidogenic peptide appears to correlate with the efficiency of fibril formation. For example, human amylin has greater toxic potency than βA in both neuronal and islet cell cultures, and human amylin shows more rapid and extensive fibril formation than βA at the same molar concentrations (unpublished results).

The amyloid fibril-binding dye Congo red prevented the neurotoxicity of βA, providing further evidence for the role of fibril formation. Congo red protected against βA neurotoxicity by a dual mechanism. For the βA-(1–40) peptide, Congo red inhibited fibril formation and also bound to preformed fibrils, rendering them nontoxic. For the βA-(1–42) peptide, Congo red protected against neurotoxicity by binding to fibrils but did not inhibit fibril formation. Congo red also inhibited the pancreatic islet cell toxicity of amylin by binding to amylin fibrils.

Congo red has been observed to inhibit conversion of the prion protein from the normal protease-sensitive form to the pathogenic protease-resistant form and to inhibit Scrapie infectivity in cell culture (25, 26). It was hypothesized that Congo red prevented the pathogenic conversion of prion by inhibiting the binding of prion to sulfated proteoglycans (27). Our observation that Congo red can inhibit βA fibril formation raises the possibility that Congo red may inhibit Scrapie infectivity by inhibiting prion fibril formation. Two mutations in the prion protein associated with familial Creutzfeldt-Jakob disease have been shown to increase fibril formation *in vitro*, consistent with a role for fibril formation in the pathogenesis of prion-related diseases (28).

We have previously shown that the pancreatic islet cell toxicity of diabetes-associated amylin is mediated by the fibrillar form of the peptide, analogous to the neurotoxicity of Fib-βA (18). Amylin is also toxic to neurons (18, 29). Furthermore, a peptide derived from the prion protein is neurotoxic in cell culture, although the role of fibril formation in this case has not yet been clearly demonstrated (30). These observations suggest that amyloid fibrils may be generally cytotoxic and may contribute to the pathogenesis of several degenerative disorders of aging. The cytoprotective effect of Congo red for βA neurotoxicity and amylin islet cell toxicity suggests that different amyloid fibrils have similar toxic mechanisms. Therefore, it is possible that agents that inhibit amyloid fibril formation or toxicity may have therapeutic efficacy for several different amyloidoses including Alzheimer disease and type-2 diabetes mellitus.

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