Reversible *in vitro* growth of Alzheimer disease β -amyloid plaques by deposition of labeled amyloid peptide

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ABSTRACT The salient pathological feature of Alzheimer disease (AD) is the presence of a high density of amyloid plaques in the brain tissue of victims. The plaques are predominantly composed of human β -amyloid peptide (β A4), a 40-mer whose neurotoxicity is related to its aggregation. Radiolabeled human β A4 is rapidly deposited *in vitro* from a dilute (<10 pM) solution onto neuritic and diffuse plaques and cerebrovascular amyloid in AD brain tissue, whereas no deposition is detectable in tissue without preformed plaques. This growth of plaques by deposition of radiolabeled β A4 to plaques is reversible, with a dissociation half-time of ≈ 1 h. The fraction of grey matter occupied by plaques that bind radiolabeled β A4 *in vitro* is dramatically larger in AD cortex ($23 \pm 11\%$) than in age-matched normal controls (<2%). In contrast to the human peptide, rat/mouse β A4 (differing at three positions from human β A4) does not affect the deposition of radiolabeled human β A4. β A4 has no detectable interaction with tachykinin receptors in rat or human brain. The use of radiolabeled β A4 provides an *in vitro* system for the quantitative evaluation of agents or conditions that may inhibit or enhance the growth or dissolution of AD plaques. This reagent also provides an extremely sensitive method for visualizing various types of amyloid deposits and a means for characterizing and locating sites of amyloid peptide binding to cells and tissues.

Alzheimer disease (AD), a widespread progressive dementia affecting a significant fraction of the elderly, is characterized by the presence in brain of numerous extracellular proteinaceous deposits called amyloid on the basis of their staining properties. The extracellular amyloid is deposited both at neuropil and vascular sites, and the density of these deposits in the cerebral cortex and blood vessels correlates positively with the degree of dementia. The principal component of both neuritic and vascular amyloid is β -amyloid peptide (β A4), a hydrophobic peptide of 39–43 amino acids that is encoded by a gene for a much larger protein termed the amyloid precursor protein. Neither the processing of amyloid precursor protein to β A4 nor the genesis of the amyloid deposits is well understood. The characteristics of β A4 deposition and the factors that affect it remain key questions in the pathology of AD (1–8).

Recent work (9–16) has suggested that β A4 has biological activities of its own, and the 42-mer or its fragments have been reported to be trophic, toxic, and/or amnesic at various concentrations. β A4 has a considerable tendency to form insoluble aggregates under various conditions (17–22) and evidence has been presented that the neurotoxicity of β A4 is related to the aggregation process (13, 14). A sequence

similarity (Table 1) between residues 25–35 of β A4 and the tachykinin neuropeptides (23–25) led to an investigation of the latter and the finding that tachykinins can block and tachykinin antagonists can mimic some of the biological activities of β A4 on rat embryonic hippocampal neurons in primary culture (15), which suggested an interaction of β A4 with tachykinin receptors. Furthermore, direct injection of β A4 (1–40) into the cerebral cortex or hippocampus of adult rats was reported (16) to have neurotoxic effects that are blocked by coadministration of the mammalian tachykinin substance P in a dose-dependent manner.

To explore the aggregation properties of human β A4, the sites at which the peptide is bound, and what factors may affect this binding, we have synthesized a ¹²⁵I-radiolabeled amyloid peptide [¹²⁵I- β A4-(1–40)] and developed a method to characterize and localize the sites with which it interacts in homogenates or thin sections of normal or AD tissue.

MATERIALS AND METHODS

Synthetic Peptides. Peptides were purchased (Bachem) or synthesized by conventional solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry in dimethylformamide. Activation was by diisopropylcarbodiimide and hydroxybenzotriazole and Fmoc groups were removed with piperidine. Peptides were purified to near homogeneity (>95%) by reversed-phase HPLC (RP-HPLC) with a gradient of acetonitrile in 0.01 M trifluoroacetic acid and a C₁₈ column. All peptides were characterized by RP-HPLC and amino acid analysis. Peptides were stored at –20°C lyophilized or in solution at 1 mM in the solvents in which they were eluted from the HPLC column with 1% 2-mercaptoethanol added as antioxidant. Under these conditions, no evidence of oxidation, aggregation, or degradation was observed over months of storage. Except as otherwise noted, all peptides are based on the human β A4 sequence (Table 1). The analogous peptide in rat and mouse [Gly⁵,Phe¹⁰,Arg¹³] β A4-(1–40) differs at three positions within this sequence. Dutch β A4, [Gln²²] β A4-(1–40), which differs at one position within this sequence, is the amyloid peptide of cerebrovascular deposits of hereditary cerebral hemorrhage with amyloidosis—Dutch type (1–4).

Radioiodination. Peptides containing tyrosine were radiolabeled by oxidative radioiodination using Na¹²⁵I and chloramine T and separated from free iodide by reverse-phase adsorption. Peptides not containing tyrosine were first acylated with the *N*-hydroxysuccinimide ester of 4-hydroxyphenylpropionic acid and then oxidatively radioiodinated as above. Labeled peptides containing methionine were then reduced from sulfoxide to native form with 2-mercaptoethanol.

Table 1. Peptide sequences

Peptide	Sequence
Human β A4	H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA ^{10 20 30 40} -OH
Rat β A4	H-DAEFGHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA ^{10 20 30 40} -OH
Dutch β A4	H-DAEFRHDSGYEVHHQKLVFFAQDVGSNKGAIIGLMVGGVVIA ^{10 20 30 40} -OH
β A4-(25-35)-OH	H-GSNKGAIIGLM-OH ³⁰
β A4-(25-35)-NH ₂	H-GSNKGAIIGLM-NH ₂ ³⁰
Substance P	H-RPKPQQFFGLM-NH ₂
Neurokinin A (substance K)	H-HKTDSFVGLM-NH ₂
Neurokinin B (neuromedin K)	H-DMHDFVGLM-NH ₂

Sequences of amyloid and tachykinin peptides. H-, free amino group; -OH, free carboxyl group; -NH₂, carboxamide. Sequences are numbered from the N termini with positions indicated by superscripts.

anol or dithiothreitol. The iodinated peptides were purified by RP-HPLC to essentially quantitative specific activity (≈ 2000 Ci/mmol; 1 Ci = 37 GBq) and stored as above at $<2 \times 10^5$ dpm/ μ l (26–28). The RP-HPLC separations employed shallow acetonitrile gradients that cleanly separate sulfoxide from reduced methionine and iodinated from noniodinated forms of the peptides. It should be noted that the radioiodinated peptides are not precisely identical in chemical structure to the native peptides.

Tissue. Brain tissue was obtained from normal and AD patients (Table 2) <14 h postmortem, frozen on dry ice after collection, and stored below -20°C until use. For filter binding studies, tissue homogenates were prepared by the method of Too and Hanley (29). Briefly, tissue was homogenized by a Polytron, washed several times with centrifugation in a buffer containing protease inhibitors, and then stored below -20°C until use. For autoradiographic binding studies, unfixed tissue was serially sectioned at $15 \mu\text{m}$ and thaw-mounted onto gelatin-coated slides. Slide-mounted sections were stored at -20°C in closed boxes over desiccant for <3 months before use. Rat brain tissue was removed immediately after sacrifice by decapitation, frozen on dry ice after collection, and stored below -20°C until use.

Homogenate Binding. Membranes (equivalent to ≈ 30 mg of tissue) were resuspended in 0.5 ml of 50 mM Tricine (pH 7.5) containing bovine serum albumin (1 mg/ml), 10 mM MnCl₂, 0.004% bacitracin, 0.0002% chymostatin, 0.0004% leupeptin, and 0.1% dimethyl sulfoxide for 30 min prior to addition of radioligand at 10 pM to 1 nM and various concentrations of unlabeled peptides in the same buffer. After incubating 2 h at room temperature, the homogenates (final volume, 0.575 ml) were filtered through glass fiber filters (Whatman GF/D presoaked for at least 2 h in bovine serum albumin at 1 mg/ml) and rinsed with 25 mM Tricine (pH 7.5). After washing twice with 25 mM Tricine (pH 7.5) at room temperature, radioac-

tivity on the filters was measured in a γ counter. The signal/noise ratio and specific binding were a function of the density of plaques in the tissue. In a typical experiment with AD tissue and 0.1 nM radioligand, 20,000 cpm (10%) of the ^{125}I - β A4-(1–40) added was bound in the absence of unlabeled peptides, and 10,000 cpm (5%) was bound in the presence of $10 \mu\text{M}$ unlabeled β A4-(1–40). During the course of the experiments described, no significant degradation or oxidation of the radioligand was detected by RP-HPLC. Tachykinin receptor binding studies were performed in a similar manner (24, 29, 30).

Autography. Tissue sections were preincubated for 30 min and incubated with radioligand for 2 h under the conditions used for homogenate binding studies except that Tris-HCl rather than Tricine was used as buffer. For estimating nonspecific binding, paired serial sections were incubated with radioligand in the presence of a 10^4 - to 10^6 -fold excess of the unlabeled peptide. After incubation with radioligand, the slides were washed with 50 mM Tris-HCl (pH 7.5; four 2-min washes at 4°C) and with distilled water (two 5-sec washes at 4°C), dried at 4°C , and stored in closed boxes over desiccant at room temperature overnight. The fully dried slides were then placed in apposition to film next to radioiodinated standards. After a 1-week exposure at -20°C ,

FIG. 1. Inhibition of specific binding of ^{125}I - β A4-(1–40) to homogenates of AD temporal cortex by various amyloid and tachykinin peptides. Each point is an average of at least three experiments, each done in triplicate; standard errors were $<10\%$ of the mean for all points. Because concentrations of β A4-(1–40) higher than $10 \mu\text{M}$ can form filterable aggregates during the course of the incubation, the asymptote of the binding curve is estimated by extrapolation. No specific binding of ^{125}I - β A4-(1–40) to homogenates of normal temporal cortex was detected. $\square$, β A4-(1–40); $\circ$, Dutch β A4-(1–40); $\triangle$, β A4-(25–35)-NH₂; $+$, substance P, neurokinins A and B, β A4-(25–35)-OH, rat β A4-(1–40), and CP-96,345.

Table 2. Patient information

Diagnosis	Sex and no.	Age, years	% plaques in cortex
AD	6F, 4M	74.4 $\pm$ 6.29 (63–84)	22.7 $\pm$ 11.2 (6.4–42.0)
Normal	4F, 3M	64.1 $\pm$ 6.12 (59–75)	<1.8 (<0.2 –1.8)

Summary of the clinicopathological features of the control ($N = 7$) and AD ($N = 10$) patients whose tissues (frontal and/or temporal cortex) were used for the present study. The percent of total grey matter occupied by plaques that bound ^{125}I - β A4-(1–40) is noted. Averages: AD, $22.7 \pm 11.2\%$; normal, $<1.8\%$ ($P < 0.01$). Diagnosis was based on clinical, neuropsychological, and neuropathological criteria or on clinical assessment by the primary physician and neuropathological examination. Data are presented as mean $\pm$ SD with the range in parentheses. Six of seven brains diagnosed normal showed $<0.2\%$ of the cortex occupied by plaques that bound ^{125}I - β A4-(1–40). No relationship between plaque density and post-mortem interval (range <1 to 13.5 h) was noted.

the film was developed, fixed, and washed. Sections were later dipped in photographic emulsion for higher-resolution autoradiography and/or counterstained by standard procedures with thioflavin S, hematoxylin/eosin, or antibodies for immunohistochemistry. Autoradiography of tachykinin receptors was performed as described (24, 30).

Time Course Experiments. Serial sections were incubated in the presence of 0.1 nM ^{125}I - βA4 (1–40) for various times. Sections incubated 2 h were then transferred to a solution containing no βA4 for further incubation. At various times, the sections were removed from the incubation medium and processed for autoradiography as above; the amount of radioligand bound to plaques was later quantified by densitometry.

RESULTS

AD vs. Normal Tissue. Whereas there was essentially no displaceable binding of ^{125}I - βA4 (1–40) to normal tissue sections or homogenates (data not shown), there was significant

specific binding to AD tissue (Figs. 1 and 2). This binding to AD tissue was not saturable. In severe cases of the disease, more than a third of the volume of the affected cortical areas examined was occupied by plaques capable of binding the radioligand; in contrast, no such deposits were detected in most of the control tissues examined (Table 2). No displaceable binding was observed when plaques were absent (i.e., in normal human or rat tissue), and autoradiography of the homogenate confirmed that the highest density of binding sites in AD tissue homogenates was on intact plaques.

Localization of Binding Sites. Visualization of the binding sites for ^{125}I - βA4 (1–40) by autoradiography showed that the ligand was deposited on parenchymal amyloid plaques and at vascular sites in AD brain. Thus in AD cerebral cortex, ^{125}I - βA4 (1–40) was deposited onto diffuse, dense, and neuritic plaques; in the latter case both the core and the halo of essentially every plaque examined were labeled (Fig. 2). Thioflavin S stained neurons bearing neurofibrillary tangles

FIG. 2. ^{125}I - βA4 (1–40) binding sites in sections of human AD brain; areas without plaques showed negligible displaceable binding of ^{125}I - βA4 (1–40). (a) Dark-field autoradiogram showing distribution of ^{125}I - βA4 (1–40) binding sites in AD temporal cerebral cortex reveals numerous plaques through the grey matter. (b) A serially adjacent section treated as in *a* except that 10^{-5} M unlabeled βA4 (1–40) was added to the incubation medium to demonstrate that the binding in *a* is displaceable. (Bar = 2 mm.) (c) Bright-field photomicrograph of immunohistochemical staining of amyloid deposits in AD temporal cortex with antisera raised against purified βA4 (anti-A4). (d) Autoradiogram of the section shown in *c* visualizing ^{125}I - βA4 (1–40) binding sites; the radioligand reveals a more extensive distribution of amyloid deposits than does the anti-A4 antibody. (Bar = 175 μm .) (e) Dark-field photomicrograph of thioflavin S (Thio S) staining in AD temporal cortex showing different types of plaques (diffuse, compact, and neuritic) visualized with the dye. (f) Bright-field autoradiogram of the same section shown in *e* visualizing ^{125}I - βA4 (1–40) binding sites. All types of plaques bind the radioligand. (Bar = 25 μm .) (g) Dark-field photomicrograph of thioflavin S staining in AD temporal cortex showing a neuritic plaque and several nearby neurons labeled. (h) Bright-field autoradiogram of the section shown in *g* visualizing ^{125}I - βA4 (1–40) binding sites. Both the core and the halo of the plaque bind radioligand; no labeling of nearby thioflavin S-positive neurons is observed. (Bar = 18 μm .)

FIG. 3. ^{125}I - βA4 (1-40) binding sites in the cerebral vasculature of human AD brain. (a) Dark-field autoradiogram showing plaques in the parenchyma and amyloid in blood vessel (BV) walls in AD temporal cortex. (b) A serially adjacent section treated as in a except that $10\ \mu\text{M}$ unlabeled βA4 (1-40) was added to the incubation medium to demonstrate that the binding in a is displaceable. (Bar = $280\ \mu\text{m}$.) (c) Bright-field photomicrograph of a section stained with hematoxylin/eosin (H&E) showing a cerebral artery. (d) Bright-field autoradiogram of the section shown in c visualizing ^{125}I - βA4 (1-40) binding sites; the highest density of binding sites is associated with the tunica media (TM). TA, tunica adventitia; L, lumen; TI, tunica intima. (Bar = $40\ \mu\text{m}$.)

outside the plaques and the plaques themselves, but deposition of the radioligand was restricted to the plaques alone. The sensitivity of detection of plaques with the radioligand far exceeds that of the antibodies we have examined (Fig. 2).

Amyloid deposits were visualized by ^{125}I - βA4 (1-40) deposition in 5–20% of intra- and extraparenchymal blood vessels in AD brain (Fig. 3). The cerebrovascular deposits were consistently labeled more intensely than cerebral plaques within the same section. In vessels seen in transverse section (Fig. 3), the deposition of βA4 radioligand was not uniform but rather was concentrated in a part of the vessel—apparently, the smooth muscle of the tunica media; the endothelium was not labeled.

Competition Binding Experiments. All experiments on tissue sections or homogenates included tubes containing identical solutions in the absence of tissue that were monitored for aggregate formation by both filtration and centrifugation. These parallel experiments showed no evidence of aggregation under the conditions described. Deposition of ^{125}I - βA4 (1-40) onto plaques in both homogenates and sections of AD cerebral cortex is significantly attenuated by excess unlabeled βA4 (1-40) and Dutch βA4 (1-40) (Figs. 1 and 2). βA4 (25–35)- NH_2 also competes at much higher concentrations, whereas the free acid βA4 (25–35)-OH has no detectable activity in this assay. Rat βA4 (1-40) had no effect on the deposition of ^{125}I - βA4 (1-40) onto plaques. No significant differences in βA4 deposition were noted between homogenates and sections of AD tissue or between neuritic and diffuse plaques.

The mammalian tachykinins (23), substance P and neurokinins A and B, and a nonpeptide NK_1 tachykinin receptor antagonist, CP-96,345 (25), did not inhibit the deposition of radiolabeled βA4 onto AD plaques over a broad range of concentration (Fig. 1).

Saturable binding sites for tachykinin radioligands selective for NK_1 and NK_3 tachykinin receptor subtypes (30) were present at similar densities in both AD and normal brain tissue (data not shown). Cell types expressing tachykinin binding sites were not identified. Unlabeled tachykinins displaced their radiolabeled analogues at nanomolar concentrations in both tissues. In contrast, there was no displacement of any of the tachykinin radioligands by βA4 (1-40), rat βA4 (1-40), or Dutch βA4 (1-40) at up to $30\ \mu\text{M}$. βA4 (1-40) also failed to displace radiolabeled tachykinins from their binding sites in rat (30) brain homogenates and sections.

Time Course of βA4 (1-40) Deposition. As quantitated by densitometry of autoradiograms, radiolabeled βA4 deposition from solution onto amyloid plaques was linear for at least several hours. When plaques onto which ^{125}I - βA4 (1-40) had been deposited for 2 h were incubated in the absence of amyloid peptide, the radiolabeled peptide dissociated from the plaques (Fig. 4). Thus deposition of ^{125}I - βA4 (1-40) onto plaques is reversible.

DISCUSSION

In Vitro Growth of AD Amyloid. Radiolabeled βA4 (1-40) binds specifically, reversibly, and with high affinity to sites in AD tissue but not normal tissue (Figs. 1–4). The observation

FIG. 4. Time course of ^{125}I - βA4 (1-40) association onto and dissociation from AD plaques in tissue sections of human AD frontal cortex. Periods as long as 4 h are shown, and a 24-h time course is shown in the *Inset*. Saturation of the film prevented accurate quantitation of the 24-h time point for association; the 24-h point shown is a lower limit. Error bars represent the SEM of at least three determinations. Analysis of the time course of dissociation yields a dissociation half-time of $\approx 1\ \text{h}$ under the described conditions.

that binding is not saturable suggests that the vast majority of the sites to which the radioligand binds are not receptors in the usual sense. Instead, the characteristics of this binding are consistent with a steady-state equilibrium between β A4 on the tissue amyloid plaques and in solution. Thus ^{125}I - β A4-(1-40) at picomolar concentrations is reversibly deposited onto AD plaques and dissociates when the plaques are exposed to a solution containing no β A4 (Fig. 4). The precise nature of the plaque component to which the radioligand binds is not addressed by these experiments.

Under these experimental conditions, the *in vitro* growth of plaques by deposition of ^{125}I - β A4-(1-40) proceeds at the lowest concentration of radioligand that can be conveniently quantitated (1-10 pM). The affinity of β A4 for the plaques is sufficiently high that deposition is a favorable process even at such dilute concentrations.

Both the classic senile plaques and the diffuse nonneuritic deposits in the cortical grey matter and the vascular amyloid deposits within the walls of the cerebral arteries are capable of *in vitro* growth by addition of β A4 from dilute solution. Use of radiolabeled β A4 detects tissue amyloid with great sensitivity and emphasizes the great density of amyloid deposits in AD vs. normal tissue (Table 2).

β A4 and Tachykinins. The tachykinin neuropeptides are defined by their C terminus, -Phe-Aaa-Gly-Leu-Met-NH₂, where Aaa is an aromatic or aliphatic residue. This conserved C-terminal sequence, including the terminal carboxamide, is required for essentially all the biological activities of the peptides in the tachykinin family.

Various tachykinin agonists and antagonists had no detectable effect on *in vitro* growth of AD amyloid (Fig. 1). Since several experiments failed to detect any interaction between β A4 and various tachykinin receptors found in normal human and AD brain and in rat brain, β A4 apparently does not interact with tachykinin receptors under these conditions (data not shown). These results are consistent with the reported structure-activity studies among the tachykinin peptide family that a C-terminal amide is required for activity (23) and argue against the hypothesis (15) that the reported biological activity of β A4 (9-16) results from its interaction with tachykinin receptors.

Aggregation of β A4 in the Absence of Tissue. Under the conditions of the tissue binding assay described above, there is no detectable aggregation of β A4 in the absence of plaques. At higher concentrations or in solvents other than those described above, β A4-(1-40) and certain of its fragments can also form aggregates (17-21) in the absence of nucleating plaques, and the radiolabeled β A4 can be used as a sensitive and convenient monitor of such aggregation. The rate of aggregation of β A4 in tissue free systems is highly dependent on experimental conditions (data not shown).

Implications for AD. The avidity of β A4 for amyloid plaques implies that once a nucleating aggregate of β A4 has formed, even extremely low concentrations of β A4 will support its growth. The reversibility of plaque growth *in vitro* suggests a steady-state equilibrium between β A4 in plaques and in solution. The *in vitro* studies described here are consistent with the hypothesis that the plaques themselves (neuritic, diffuse, and vascular) can grow *in vivo* in the presence of traces of β A4. A similar situation *in vivo* would imply that an alteration in the processing or degradation of β A4 that increased the extracellular concentration of β A4 could play a critical role in the genesis of AD (1) and that plaque growth in the brain may be reversible. The spontaneous aggregation of β A4 under certain conditions is likely to be related to the early nucleating events of plaque formation and thus to the neurotoxicity of β A4 (13-22) and the pathology of the disease. The use of radiolabeled β A4 provides a convenient model of these processes for further investigation of the role of β A4 in AD. The present study suggests a

sensitive and quantitative assay for evaluation of agents and conditions that may alter the growth of amyloid plaques and aid in the search for effective treatments of AD.

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