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Monoclonal antibodies inhibit *in vitro* fibrillar aggregation of the Alzheimer β-amyloid peptide

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ABSTRACT **The β-amyloid peptide, the hallmark of Alzheimer disease, forms fibrillar toxic aggregates in brain tissue that can be dissolved only by strong denaturing agents. To study β-amyloid formation and its inhibition, we prepared immune complexes with two monoclonal antibodies (mAbs), AMY-33 and 6F/3D, raised against β-amyloid fragments spanning amino acid residues 1–28 and 8–17 of the β-amyloid peptide chain, respectively. *In vitro* aggregation of β-amyloid peptide was induced by incubation for 3 h at 37°C and monitored by ELISA, negative staining electron microscopy, and fluorimetric studies. We found that the mAbs prevent the aggregation of β-amyloid peptide and that the inhibitory effect appears to be related to the localization of the antibody-binding sites and the nature of the aggregating agents. Preparation of mAbs against "aggregating epitopes," defined as sequences related to the sites where protein aggregation is initiated, may lead to the understanding and prevention of protein aggregation. The results of this study may provide a foundation for using mAbs *in vivo* to prevent the β-amyloid peptide aggregation that is associated with Alzheimer disease.**

Experimental evidence that β-amyloid peptide (βA4), the hallmark of Alzheimer disease (1, 2), has opposing neurite-promoting and neurotoxic properties that are related to peptide aggregation forms (3–5) has focused the development of appropriate therapeutic approaches toward reducing or eliminating the extent of amyloid fibrillar deposition in the brain (6–8). Amyloid insolubility has been one of the most insurmountable problems in the initial characterization of the constituent proteins of the isolated plaque cores from brains affected by Alzheimer disease. Strong denaturing conditions, such as high concentrations of urea, guanidine·HCl, or extreme pH, are required to break and dissolve such aggregates (2, 9).

Under physiological conditions, the synthetic βA4 adopts an aggregated form and also shows a change from a neurite-promoting to a neurotoxic effect on hippocampal neurons (3–5, 10). Aggregation of βA4 has been shown to depend on pH, peptide concentration, temperature, and time of incubation (11). The so-called pathological chaperones (12), as well as such metal ions as Zn^{2+} and Al^{3+} (13–15) that have been proposed as "risk factors" for Alzheimer disease, accelerate the β-amyloid cascade aggregation.

The availability of monoclonal antibodies (mAbs) that bind to a specific antigen at distinct, well-defined sites has led to a better understanding of how highly specific antigen–antibody interactions can affect antigen behavior. Like the ubiquitous chaperones (16), mAbs raised against specific native antigens assist in antigen refolding (17–19) by recognizing incompletely folded epitopes and inducing their native conformation. By appropriate selection, mAbs have been isolated that bind to predefined locations on certain protein or peptide molecules without inhibiting their biological activity (20, 21).

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In this study, we investigated the ability of mAbs against βA4 to suppress the *in vitro* aggregation of βA4 via immunocomplexation. We used a sandwich ELISA method and fluorescence monitoring, as well as electron microscope studies, to characterize the effect of two different mAbs on β-amyloid formation.

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal antibodies raised against synthetic βA4-(1–40) were obtained from Boehringer Mannheim. Aggregation of βA4-(1–40)-peptide was monitored by using two commercially available mAbs raised against the respective peptides 8–17 and 1–28 of βA4 anti-human β-amyloid mAb 6F/3D (Accurate Chemicals) and mAb AMY-33 (Zymed).

βA4 Aggregation and Immunocomplexation. Synthetic βA4-(1–40) was obtained from Sigma. For *in vitro* induced aggregation, the reaction mixture tubes containing 200 μl of an aqueous solution of βA4 (2.5×10^{-5} mM), heparan sulfate (50 nM), and/or chloride metal solutions (10^{-3} M at pH 6.5) or βA4 by itself were incubated for 3 h at 37°C. Aggregated β-amyloid samples were removed by centrifugation for 15 min at 15,000 × *g*. To determine the soluble βA4 the supernatants were then incubated for another 60 min with an excess of mAb AMY-33 and/or 6F/3D (as determined from the following set of experiments) to produce immunocomplexed βA4. In another set of experiments, mAb at equimolar antibody/antigen concentrations were added to the reaction mixtures before the first incubation period of 3 h at 37°C. The amount of βA4 left in solution under the various conditions employed can be determined from both sets of experiments.

βA4 aggregation was followed by three approaches:

(*i*) ELISA. The ELISA coating procedure, with Eupergit-C-containing epoxy groups (Rohm, Germany), using beads or paper as the solid phase, has been described (22, 23). In the present study, rabbit anti-β-amyloid-(1–40) antibody (100 ng/well) was covalently attached to epoxy-coated Microtiter plates for 16 h at 4°C. After the plates were washed with phosphate-buffered saline (PBS) containing 0.005% Tween 20, the residual epoxy groups were blocked by adding 1% fat low-fat milk. Before use, the plate was thrice washed with PBS/Tween 20 and then dried. The soluble immunocomplex of anti-β-amyloid/βA4 obtained as described above was added to the plates for 1 h at 37°C, and bound mAb was measured by excess of horseradish peroxidase-labeled goat-anti-mouse antibody (Bio-Rad). Degradation of the *O*-phenylenediamine substrate by HRP was monitored at A_{495} according to manufacturer's instructions by using an ELISA reader. The amount of mAb bound was assumed to be proportional to the amount of soluble amyloid peptide that remains in the reaction tube after incubation at the various aggregation conditions specified. The data represent the mean of three replicates. The standard deviations of the intraassay and interassays were >5% in all cases.

Abbreviations: mAb, monoclonal antibody; βA4, β-amyloid peptide.
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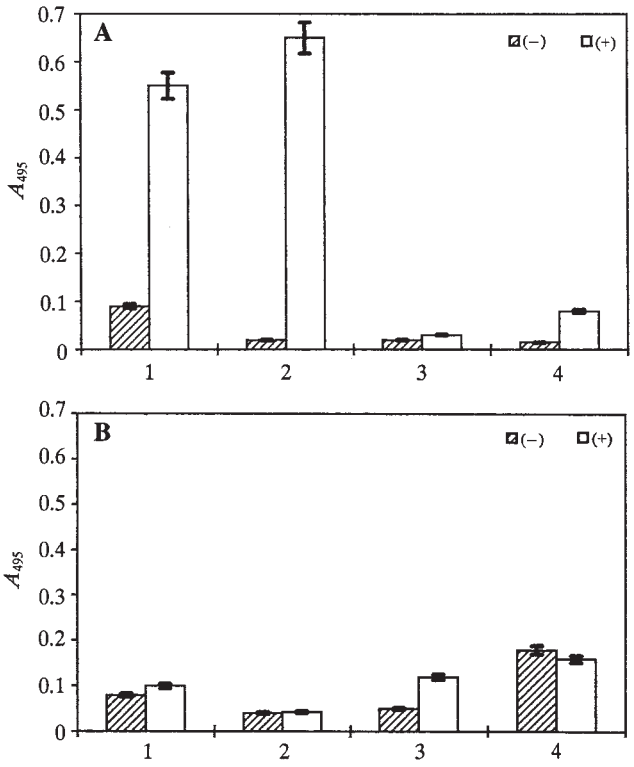

FIG. 1. Relative amounts of residual soluble βA4-(1-40) in the absence (−) or in the presence (+) of mAb AMY-33 (*A*) or 6F/3D (*B*), as measured by ELISA (as described in *Materials and Methods*). 1, βA4; 2, βA4 plus 50 mM heparan sulfate; 3, βA4 plus 10^{-3} M $AlCl_3$; 4, βA4 plus 10^{-3} M $ZnCl_2$.

(*ii*) Electron microscopy. Negatively stained amyloid fibrils were prepared by floating carbon-coated grids on aqueous peptide solutions (1–2 mg/ml) and air drying. Fibrils of β-amyloid, either alone or immunocomplexed to mAb AMY-33 (molar ratio of 4:1) for 3 h at 37°C, were negatively stained with aqueous (2% wt/vol) uranyl acetate and then visualized by using a JEOL model 1200 EX electron microscope operated at 80 kV with a magnification of 25,000.

(*iii*) Fluorometry. Fluorometric analysis of soluble β-amyloid peptide and the immunocomplex with AMY 33 (molar ratio of 4:1) stained with thioflavin T (Sigma) was performed by a standard method (24). Fluorescence was measured using a Perkin–Elmer model LS-50 fluorimeter at an excitation wavelength of 450 nm and an emission wavelength of 482 nm. The aggregation reaction was followed for 7 days at 37°C.

RESULTS

Effect of Immunocomplexation on the *in Vitro* Aggregation of βA4. mAbs were added to the reaction mixture before or after the incubation of synthetic βA4 under experimental aggregation conditions in the presence of heparan sulfate and/or such metal ions as Zn^{2+} and Al^{3+} at recommended concentrations (13–15, 25), as described in *Materials and Methods.* The results shown in Fig. 1*A* indicate that mAb AMY-33, which recognizes an epitope spanning amino acid residues 1–28 of βA4, inhibited the aggregation of peptide in the presence or absence of heparan sulfate. No inhibitory effect on metal-induced amyloid aggregation was seen under the same experimental conditions. The mAb 6F/3D, which recognizes an epitope located between residues 8 and 17 of βA4, slightly interfered with Zn^{2+}-induced aggregation but had no effect on the self-aggregation induced by other aggregation-inducing agents (Fig. 1*B*).

mAb-Induced Conversion of β-Amyloid from Fibrillar to Nonfibrillar Conformation. Electron microscopy of negatively stained β-amyloid and its immunocomplex with mAb AMY-33 (Fig. 2) revealed that even at a low peptide to antibody ratio,

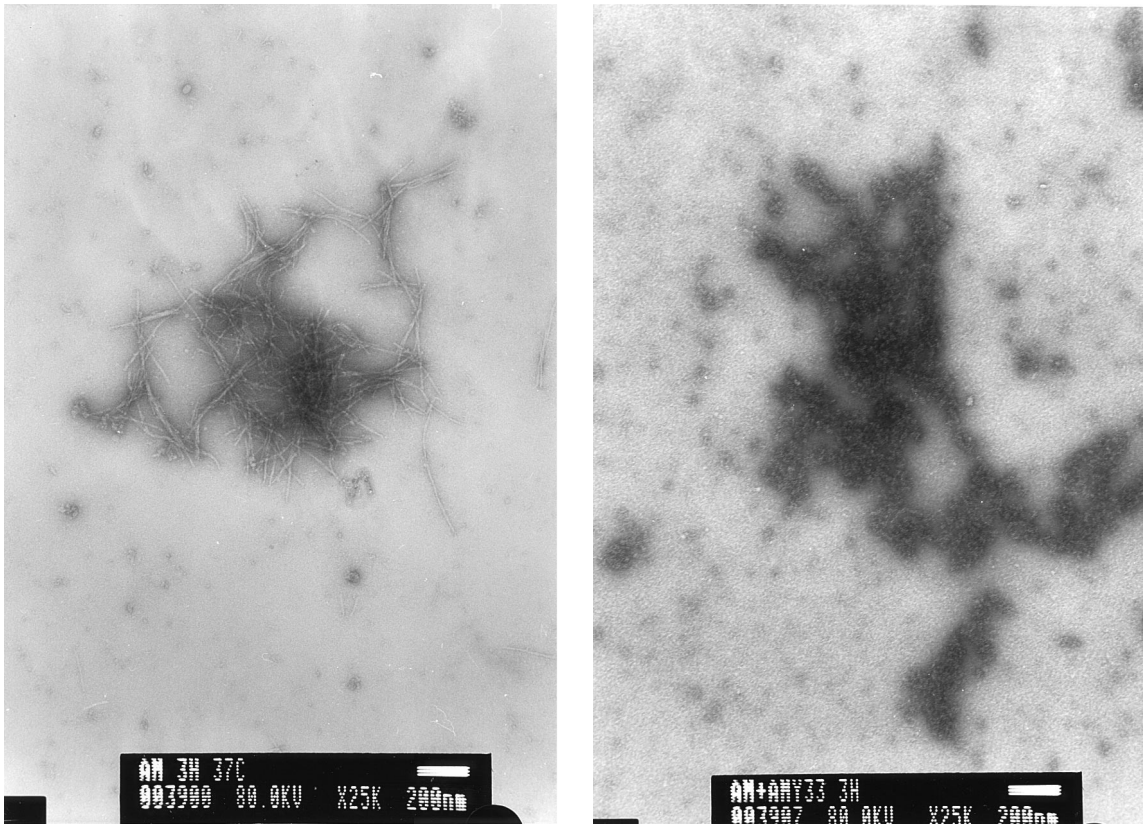

FIG. 2. Electron micrographs of β-amyloid aggregates in the absence (*Left*) or presence (*Right*) of mAb AMY-33. (Bars = 0.2 μm.)

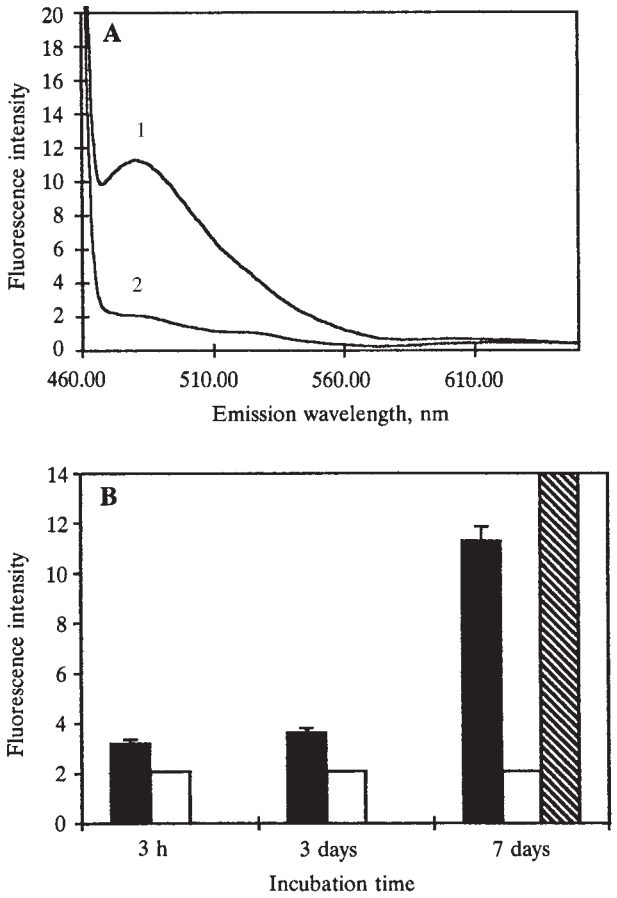

FIG. 3. Thioflavin T-based fluorometric assay of β-amyloid fibril formation. (*A*) Emission spectra of thioflavin T bound to fibrillar βA4 alone (curve 1), or in the presence of the immunocomplex of βA4 with antibody AMY-33 (curve 2). (*B*) Increase in thioflavin T fluorescence bound to βA4 (■) as a function of incubation time of the peptide measured after 3 h, 3 days, and 7 days. Immunocomplexation with mAb AMY-33 prevents the increase in thioflavin T fluorescence (□), while unrelated antibody did not interfere with its fluorescence (▨).

fibrillar β-amyloid was converted to an amorphous state. Thioflavin T, a suitable probe for detecting the fibrillar aggregation of βA4, confirmed the electron microscopy results. Dilution of the peptide directly from water into dye-containing buffer had no effect on dye fluorescence. In the presence of aggregated βA4-(1–40), however, a change occurred in the excitation spectrum of thioflavin T, manifested as a new peak at 450 nm that was not seen with the free dye (see Fig. 3*A* for a representative emission spectrum). Aggregated βA4 enhances the fluorescence emission of thioflavin T at 482 nm as a function of incubation time. Adding mAb AMY-33 (at a ratio of 4:1) to the βA4(1–40) solution before exposure to 37°C prevented the increase in fluorescence at 482 nm, whereas the addition of unrelated antibody did not interfere with the fluorescence peaks (Fig. 3*B*).

DISCUSSION

In suppressing β-amyloid aggregation, mAbs AMY-33 and 6F/3D, which recognize different epitopes of the βA4 chain, exhibited a selective chaperone-like activity. The immunocomplex of mAb AMY-33 and βA4 not only prevented self-aggregation of βA4 but also the aggregation that was induced in the presence of heparan sulfate, which is thought to affect only the aggregation of preexisting amyloid fibers (26). The inhibitory effect was related to the localization of the antibody-binding sites and to the nature of the aggregating agents. The results of negative-staining electron microscopy revealed that even at low concentrations of mAb AMY-33 only amorphous aggregates are formed. The ELISA measurements indicated that increasing the concentration of mAb AMY-33 to equimolar antigen/antibody ratios maintained βA4 solubility. The diffuse and amorphous conglomerates of βA4 deposits that were not detectable by thioflavin T fluorometry or Congo red staining are not supposed to be associated with neuritic pathology (24).

The mAb AMY-33 did not exhibit a similar inhibitory effect on metal-induced amyloid aggregation. The slight interference with Zn^{2+}-induced βA4 aggregation that occurred using mAb 6F/3D may be due to the partial solvation effect of already aggregated βA4. Aluminum-induced aggregation of βA4 is distinguished from that induced by zinc in terms of its role, extent, pH, and temperature dependence (13). Although the precise site of metal-ion interaction with βA4 has not yet been determined, several residues in the βA4 chain are candidates for metal binding. The βA4 residues His^{13} and His^{14} may be implicated in fibril formation, and, conceivably, His^{14} might remain available for intermolecular electrostatic interactions between antiparallel chains (25). The site defined by Val^{12}-His^{13}-His^{14}-Glu^{15}-Lys^{16}-Leu^{17} is thought to provide the cationic binding sites that are exposed on the same face of the peptide β-sheet (25).

Because βA4 has been shown to be physiologically produced in a soluble form in normal individuals (27, 28), the aggregation of soluble βA4 into insoluble amyloid fibrils is believed to be a crucial step in the pathogenesis of Alzheimer disease. Therefore, to reduce or eliminate the extent of pathological protein depositions in the brain, much effort has been focused on developing potent and selective inhibitors of β-amyloid aggregation (6–8). Preparing mAbs against "aggregating epitopes," identified as sequences related to the sites where protein aggregation is initiated, may provide a tool for preventing the phenomenon of protein aggregation. In previous studies, we showed that appropriate mAbs interact at strategic protein-folding-initiation sites, leading to a considerable refolding effect of the already clustered epitopes (19).

The ability of mAbs and their respective genetically engineered antibody fragments to suppress *in vivo* aggregation of βA4 in cultured PC-12 neural cells is under investigation.

Recent advances in antibody engineering have enabled not only the manipulation of antibody genes but also the reshaping and designing of antibody molecules for intracellular expression (29, 30). The observation that active antibodies can be targeted to specific subcellular compartments by linkage to the appropriate signal sequences raises the additional possibility that single-chain antibodies can find targets inside the cell, providing a different class of active molecules for gene therapy. Recent success in producing congophilic amyloid deposits in rat brains (31) and the murine Alzheimer model system (32) provide the rationale to study the effects of such antibodies against β-amyloid in brain tissue. These models can provide the foundation for potential therapeutic approaches targeted at the fibrillar β-amyloid accumulation in Alzheimer disease.

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