

Targeting Generation of Antibodies Specific to Conformational Epitopes of Amyloid β -Derived Neurotoxins

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Abstract: Individuals with early Alzheimer's disease (AD) suffer from a selective and profound failure to form new memories. A novel molecular mechanism with implications for therapeutics and diagnostics is now emerging in which the specificity of AD for memory derives from disruption of plasticity at synapses targeted by toxic A β oligomers (also known as ADDLs). ADDLs accumulate in AD brain and constitute long-lived alternatives to the disease-defining A β fibrils deposited in amyloid plaques. The AD-like cellular pathologies induced by ADDLs suggest their impact could provide a unifying mechanism for AD pathogenesis, explaining why early stage disease is specific for memory and accounting for major facets of AD neuropathology. Discovery of these new toxins has provided an appealing target for disease-modifying immunotherapy. For optimal protection against these toxins, antibodies should bind to the pathological oligomers without being depleted by their monomeric subunits, which are rapidly generated by membrane protein turnover. A solution to this problem is likely to come from the continued development of conformation-specific antibodies, as described here. Prototype conformation-specific antibodies, not yet in the clinic, have been introduced and utilized in multiple applications for their ability to bind with high specificity and affinity to ADDLs. It can be anticipated that further development of such antibodies for use in clinical trials will come in the near future.

Keywords: Alzheimer's disease, ADDL, oligomer, synapse, neurodegeneration, CNS neurons, disease-modifying therapeutics, diagnostics.

Alzheimer's disease (AD) is an illness caused by endogenous neurotoxins. Like 30 other proteinopathies, these neurotoxins arise from physiological proteins that mis-fold or mis-assemble [1-3]. The presence of a pathological species demonstrably absent from healthy individuals provides a compelling target for immunotherapy. Elimination of these toxins may not only stop disease progression, but may actually reverse the dysfunction in mild cognitive impairment and early-stage AD. An important goal is to establish the correct identity of the toxins and generate the means to neutralize or eliminate them by using antibodies optimally directed against unique conformational epitopes.

The potential of immunotherapy as a viable treatment for AD first became evident with the extraordinary breakthrough of Schenk and

colleagues who demonstrated that active vaccination causes elimination of plaques. Immunization of transgenic (Tg) AD mouse models with fibrillar amyloid beta protein (A β) reduced A β deposits in older mice and prevented accumulation in younger mice, when immunization occurred prior to plaque formation [4, 5]. Vaccination improved neuritic structure and protected against cognitive decline as measured by the Morris water maze [6, 7]. Passive immunization with A β antibodies also was shown to reduce amyloid plaque burden [8-17].

The A β plaques that were removed by immunization were originally considered the primary target for therapeutics. The major component in plaques is fibrillar A β [18], a potent cytotoxin capable of attaching to neurons and triggering death cascades [19-21]. Increased production of A β has been linked to familial AD mutations in the amyloid precursor protein (APP), from which A β is cleaved, and in presenilins 1 and 2, which participate in its cleavage [22, 23]. These genetic links to AD combined with fibrillar toxicity helped generate strong support for the original cascade hy-

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pothesis: AD is a consequence of brain neuron death caused by large insoluble A β fibrils [21, 24]. This powerful hypothesis, which dominated thinking about AD therapeutics for nearly 20 years, helped to focus attention on A β fibrils as the primary toxin to be eliminated by immunotherapy.

DISEASE-SPECIFIC ANTIBODIES

New Concept of the Right Target: Soluble A β Oligomers. Since Schenk's discovery, there has been a radical change in the concept of what constitutes the correct immuno-target. Although evidence still strongly implicates A β involvement in AD, the abundance of A β fibrillar plaques has proven to be a poor pathological correlate of dementia [25-28]. Evidence suggesting an alternative form of A β toxin was first provided by Finch and colleagues, who reported that blocking fibril formation with apoJ did not eliminate toxicity toward the neuron-like PC12 cell line [29]. Following this important clue, Lambert et al. [30] determined that these solutions contained small globular A β oligomers that were potent CNS neurotoxins. The oligomers, which have been named A β -derived diffusible ligands (ADDLs) to underscore that they represent a new type of toxin structurally and mechanistically distinct from fibrils and protofibrils, caused death in selected neuronal populations following chronic exposure. In addition, following acute exposure, ADDLs inhibited long term potentiation, an established paradigm for synaptic plasticity and memory formation. Toxic soluble A β oligomers, which can be generated spontaneously without apoJ [27, 31-33] and are not detected by traditional measurements of plaque load, offered a basis for reconciling A β involvement in AD pathogenesis with the poor correlation between fibrillar amyloid and disease progression.

Oligomers have been detected *in vitro* and in brain since the early 1990's but only after 1998 [30] have they been recognized as putative neurotoxins responsible for dementia. Alternative forms of soluble oligomers have emerged as possible disease-causing toxins. These include protofibrils [34], dimers [35], trimers [35], Abeta*56 [36], globulomers [37], micelles [38, 39], prefibrillar aggregates [34], amylospheroids [40], TA β [41], paranuclei [42], and annular protofibrils [43]. These oligomers have been formed under varied

conditions and display different toxic activities. It is not yet clear how many stable entities truly exist or the extent to which they represent readily interconvertible structures. The term ADDL is a generic name referring to all soluble globular oligomers with neurotoxic activity.

Oligomers have now been extensively investigated (For reviews, see [27, 44-46]), leading to a new hypothesis for the mechanism of AD. Early memory loss is considered the consequence of synapse failure, not neuron death, and the disease-causing molecule is an A β oligomer, not the fibrillar A β of amyloid plaques [47]. It follows that immunotherapy would be most effective if directed against particular A β oligomers [27]. Support for this goal and the oligomer hypothesis comes from investigations of mouse models of early AD which develop plaques and memory dysfunction as they age. The animals show significant improvement in memory function when injected with antibodies against A β , with recovery evident in as little as 24 hours [48]. Recovery in the mice occurs without any decrease in plaque burden [48, 49]. These findings further substantiate the concept that fibrils are not the only neurotoxic form of A β and likely not even the most significant form in AD. Neuron damage and memory loss in AD is now widely attributed to pathogenic oligomers [50].

Introduction of Assembly-Specific Antibodies.

All oligomers derive from the same monomeric subunit. The goal is to develop specific antibodies that target and neutralize only the toxic assemblies. Antibodies that target linear epitopes in A β are less desirable because of their inability to discriminate physiological from pathological species. To distinguish oligomers from monomers, or from fibrils and natively folded precursor proteins, antibodies with conformational specificities are needed. Such conformation-dependent antibodies are becoming powerful tools for a wide range of research applications and can be expected to show clinical promise.

Parallel efforts by two groups pioneered development of conformational antibodies that target pathological species, leading to clinical validation of the oligomer hypothesis through detection of oligomers in AD brain slices and soluble extracts.

In the first instance, using A β fibrils as immunogen, Glabe's group produced a conformation-dependent polyclonal antibody that recognized A β oligomers and fibrils without detecting monomer [51], but this antibody received only limited use. Once oligomers were identified as relevant AD toxins, however, greater focus was placed on development and use of antibodies that distinguish oligomers from other A β species. Klein and colleagues used ADDLs as immunogen while Glabe and colleagues used A β 40 C-terminal gold colloids to generate polyclonal antibodies extremely selective for assembled forms of A β . ADDLs were found to be potent immunogens and generated antisera with roughly 1000-fold higher affinity for oligomers compared to monomers. These two sets of polyclonal antibodies were pivotal in validating the oligomer hypothesis, showing a striking disease-dependent presence of oligomers in human brain. Significantly, the ADDL-generated antibody also was used to establish the structural equivalence of brain-derived oligomers with synthetic oligomers used in toxicology experiments.

Polyclonal antibodies generated by ADDLs (M93 and 94) and A β 40 C-terminal gold colloids (A11) showed similarities and a few significant differences [31, 52]. First, these antibodies showed that distribution of oligomers in AD brain sections is distinct from fibrillar amyloid deposits detected by thioflavin-S staining. A feature of the staining seen with the ADDL-generated antibody is an early-stage perineuronal pattern attributed putatively to synaptic binding. Both A11 and the ADDL-generated antibodies also effectively block oligomer toxicity *in vitro*. Differences between A11 and ADDL-generated antibodies are evident in target specificity. The ADDL-antibodies selectively bind oligomers and fibrils with little monomer binding, whereas A11 binds only to oligomers without binding to fibrils. The most striking difference is that A11 binds to oligomers of multiple fibrillogenic, disease-associated proteins [52]. These include oligomeric and prefibrillar aggregates from α -synuclein, islet amyloid polypeptide, polyglutamine, lysozyme, human insulin, and prion peptide 106-126. ADDL-generated antibodies do not. The conformationally distinct epitope of A11 thus appears to be sequence independent,

leading to the hypothesis that there exists a structural element common to oligomers. Because of its unusual specificity for a generic conformational epitope, A11 has been useful for examining the localization and pathogenic significance of oligomers for other types of amyloid-related degenerative diseases. The anti-oligomer antibody has been shown to stain diseased tissue associated with desmin-related cardiomyopathy [53] and age-related macular degeneration [54]. These findings suggest there may be more diseases remaining to be recognized that accumulate amyloid oligomers.

Mechanisms for Oligomer Discrimination.

How these antibodies discriminate between a monomeric precursor and an oligomeric product has not been rigorously investigated. Speculatively, antibody recognition based on conformation-dependent targets can be envisioned as occurring via two main alternatives: 1) An epitope is formed when the monomeric subunits interact within the oligomer to generate a uniquely folded conformation, or 2) Identical linear sequences of monomers within the same oligomer provide two epitopes for binding to a single antibody. In the first case, Fab fragments would be equally effective as the whole antibody, but in the second case Fab fragments would no longer have the high binding energy provided by two sites, and thus would bind similarly to monomers and oligomers.

Oligomer Preparations as Antigens. The ongoing generation of conformation-dependent antibodies with disease-appropriate selectivity has been pursued using various protocols for antigen preparation. A number of aggregated A β species have been used as immunogens including oligomeric A β 40 preparations [55], protofibrils produced from A β 42 (E22G) [Arctic APP mutation [56]], and A β 42 globulomers (short for globular oligomers) and their thermolysine truncated A β 20-42 counterpart [37]. These protocols have generated antibodies with a variety of useful specificities (Table 1). With respect to use of ADDLs as immunogen, a strategy was implemented to generate a series of monoclonal antibodies that, like the original polyclonals, were conformation dependent [57, 58]. Clones were screened initially by dot immunoblot for binding to synthetic ADDLs or AD brain pellets enriched

Table 1. Overview of AD-Related Conformation-Specific Antibodies

Immunogen	Specificity	Uses	Refs.
ADDLs	Oligomers, protofibrils, fibrils	IF ¹ , WB ² /dot blot (human), neutralize toxicity, test oligomerization inhibitors,	[31, 96]
	Higher order oligomers, fibrils, A β 28	WB/dot blot (human), IHC ³ (human), IF, block ADDL binding, toxicity & ROS ⁴ increase	[57]
	ADDLs	IF, block ADDL binding & spine loss, IHC (human)	[58]
A β 40 oligomers	A β 40 oligomers	IHC (human)	[55]
A β 42 and A β ₂₀₋₄₂ globulomer,	A β 42 globulomer & A β ₂₀₋₄₂ oligomer	IHC (human), WB/dot blot (human / mouse), IHC (globulomer injected brain)	[37, 71]
	A β 42 & A β ₂₀₋₄₂ globulomers	IP ⁵ (human/mouse), block A β globulomer binding, passive immunization \pm	[71, 72]
Peroxynitrite treated A β 40	Oligomeric and fibrillar A β (N-terminus)	IP (oligomers), IEM ⁶ (fibrils), IHC (human/mouse), passive immunization \pm	[60]
A β 40 soluble oligomer mimic (thioester/gold colloids)	Oligomeric intermediates \geq 40kDa; non-A β oligomers and protofibrils	IF (human), inhibit A β and non-A β oligomer neurotoxicity	[52]
A β 42 Arc protofibrils	Protofibrils, fibrils	ELISA (A β protofibrils in biological samples)	[56]
Stabilized A β 40 protofibrils	A β 40 monomers, stabilized protofibrils, fibrils		[59]
A β 42 fibrils	Fibrils, soluble fibrillar oligomers; α -synuclein fibrils, IAPP fibrils	IHC (human), WB (human), IAPP ⁷ staining (mouse pancreatic tissue)	[70]
A β 40 fibrils	A β 40 fibrils, amyloid fibrils, unrelated aggregates		[103]
PalmA β 15 & PEG-A β 16 (active)	β -sheet amyloid sequences	Restore cognitive memory, reduce brain amyloid	[61]
A β 28 or A β 42 crosslinked to ovalbumin	SDS-resistant A β aggregates	IP /WB (amyloidogenic fragments of APP)	[51]
Trx(A β 15) ₄ (active)	A β 42 oligomers & fibrils, Ser ⁸⁴ -TTR oligomers & fibrils	IHC (human), A β pathology clearance (mouse)	[62]
(A β 1-11) ₂ -PADRE	A β 42 monomer, oligomers, fibrils, APP	IHC (human), block toxicity, inhibit fibril assembly, disaggregate fibrils, passive immunization \pm	[63]
	Insoluble A β 40/A β 42, A β 15	IHC (human), reduce plaques	[64]
Antibody domain (camelid library)	A β 40 fibrils	IHC (human/culture), inhibit fibril formation	[65]
	Oligomeric A β	IHC (human), inhibit fibril formation, prevent neurotoxicity \pm	[66]
A β 42 oligomer (phage library)	A β 42 oligomers	Inhibit aggregation, block toxicity (neuroblastoma), WB /dot blot (human)	[67]
Aggregated A β 40 mimic	A β oligomers	Oligomer ELISA (human)	[78]

¹IF (Immunofluorescence).²WB (Western blot).³IHC (Immunohistochemistry).⁴ROS (Reactive oxygen species).⁵IP (Immunoprecipitation).⁶IEM (Immuno-electron microscopy).⁷IAPP (Islet amyloid polypeptide).[±]In Tg mice.

in fibrils. Positives clones were then tested by Western immunoblot to confirm and further define binding specificity. This strategy generated several monoclonal antibodies that distinguish between soluble extracts from AD and control brains and that can neutralize the effects of both synthetic ADDLs and AD brain extracts on neuronal cell cultures [57]. Thirty-eight clones were saved for future expansion while the properties of six were investigated in detail. Most surprising was the production of an antibody that recognized a novel pathological form of A β unique to reactive astrocytes.

In addition to the conventional injection of antigens in multiple forms, strategies have been developed to overcome problems such as antigen conformation instability, immune tolerance, and autoreactive T-cell responses. Various approaches and the problems they address are discussed below.

Conformation Stabilization, Immune Tolerance, Autoreactive T-Cell Responses. To ensure conformational integrity of antigens, groups have employed calmidazolium to form stable protofibrils [59] and peroxyxynitrite to form oligomers [60]. When used as antigens, these stabilized species elicit an immune response that produces antibodies to a wide range of A β 40 conformations [59, 60]. To overcome the issue of immune tolerance and prevent pancreatic plaque build-up, liposomal vaccines have been created using tetrapalmitoylated A β 15 (predominantly β -sheet) or polyethylene glycol-linked A β 16 (random coil conformation) peptides, both resulting in conformation-dependent antibodies while eliminating plaque build-up in the pancreas [61]. To prevent autoreactive T-cell responses two approaches have been developed. One promising antigen incorporated a 4-fold A β 15 repeat bearing a 3aa linker arranged in tandem with the display site of bacterial thioredoxin [62]. Another antigen comprised 2 copies of A β 11 fused with the promiscuous nonself T cell epitope, PADRE, known to completely eliminate autoreactive T cell responses while retaining humoral immune responses [63, 64]. Both antigens produced conformation-dependent antibodies while preventing T-cell responses.

Phage Libraries. Single-chain binding domain antibodies, generated by panning phage libraries,

provide an alternative to conventional immunization. Biopanning of camelid phage libraries resulted in isolation of VHH-domains. These domains represent the smallest, naturally occurring antigen binding sites since they do not contain a light chain and their specificity is encoded within a single polypeptide chain. The expressed resultant domains were specific for A β 40 fibrils [65] or oligomeric A β [66]. Genetic fusion of the antibody domain molecule to *E. coli* alkaline phosphatase produced a fusion protein dimer with divalent binding characteristics and improved fibril affinity [65]. A randomized, single framework phage display library panned with oligomeric A β identified a single-chain variable domain (scFv) used to produce a monoclonal antibody that bound oligomeric A β , but not monomer or fibrillar forms [67]. This alternative method successfully produces conformation- and amyloid-specific antibodies which may be useful in avoiding the inflammatory responses of conventional antibodies¹.

Antibody Summary. Examples of antibodies that have been produced by the above strategies are illustrated in Table 1, along with other information summarizing specificity and applications. Evaluation of specificity ultimately requires the production of consistent test species (oligomers, protofibrils, fibrils) that are stringently evaluated for size and structure, including retention of antigen conformation during assay. Solid phase assays have comprised dot immunoblots, Western blots from native PAGE and SDS-PAGE, and enzyme linked immunosorbent assays (ELISAs). Importantly, competition-based assays have provided validation of antigen recognition in solution [57]. Although enviable specificity has been achieved in many of the cases described above, what is still missing and greatly desired are antibodies that reliably recognize unique A β assembly states, e.g. trimers, 12mers, doughnuts, etc. The ability of a neutralizing antibody to target a single conformational species would yield valuable information regarding a structure's pathogenic role, a matter of current controversy.

¹ Four conformation-dependent scFv antibodies were generated by phage display that specifically target toxic A β oligomers with epitopes different from those previously described (Wang XP, *et al.* FEBS Lett. Epub Jan 20, 2009.

USES OF CONFORMATION-SPECIFIC ANTIBODIES

Conformational antibodies have already proven to be powerful tools for AD research, with potential for therapeutics and diagnostics. Utilized in numerous assays to detect and/or measure toxin levels in human and Tg-mice samples [31, 37, 52, 57, 58, 60, 62-65, 68-71], these antibodies are providing new insights into the nature of the toxins and their pathogenic mechanisms. Employed to neutralize toxic species [31, 52, 57, 63, 65], conformational antibodies are also used in preclinical immunotherapy strategies [60, 61, 63, 72], and in diagnostic assay development [73, 74].

Clinical Relevance. Using ADDL-generated conformational antibodies, highly sensitive dotblot immunoassays have been developed that detect femtomole levels of ADDLs [68, 69, 75, 76]. These assays have provided a means to measure small amounts of ADDLs within a large monomer-containing milieu and thereby assess their clinical relevance in AD. Examination of soluble extracts from human brain tissue revealed that ADDLs showed a striking elevation in tissue from diagnosed AD cases, exceeding controls by as much as 70-fold [69]. Results show that A β oligomer accumulation is high in extracts of frontal cortex but only marginally above background in cerebellum [77], a pattern consistent with other aspects of AD pathology. Similar AD-dependent accumulation of oligomers was measured using the A11 antibody [52]. Two-dimensional electrophoresis showed a remarkable correlation in both size and isoelectric point between synthetic and AD brain-extract ADDLs [69]. The predominant oligomer identified by these analyses was a 12mer (~54 kDa) of the A β 42 peptide. The structural equivalence of the synthetic and naturally-occurring ADDLs is in harmony with their similar impact on nerve cell biology [69, 77]. The finding of 12mers in AD brain was confirmed by subsequent experiments with Tg mice. An apparently equivalent assembly first appears developmentally at an age coinciding with memory dysfunction [36]. The mouse molecule has been named A β *56. Anti-ADDL antibodies have also been paired with ultra-sensitive nanotechnology to measure ADDL concentrations in human CSF which also showed a striking AD-dependent accumulation [73, 74]. Occurrence of

ADDLs in brain extracts and CSF of Alzheimer's individuals has established the clinical relevance of these potent neurotoxins.

Conventional methods featuring conformational antibodies include ELISA and immunoblot. A sandwich ELISA using a conformational antibody for both capture and detection was developed to quantify changes in A β protofibril levels. Results from brain extracts showed that Tg-mice (Arctic mutation) have ~ 3.5-fold higher protofibril levels than control mice [56]. Similar sandwich ELISAs were developed to quantify A β oligomers in soluble extracts from human and Tg-AD mouse brains [37, 78]. Levels of specific A β oligomers in Tg-mouse brain were found to be age-dependent and correlate with plaques [37]. Femtomole immunodetection of A β oligomers was achieved using conformational antibodies in a dot immunoblot [68]. This assay was also used to measure the region-specific accumulation of oligomers in Tg mouse brain. When a dot immunoblot was utilized to measure A β oligomers in soluble human brain extract, Gong *et al.* [69] demonstrated a 70-fold increase in AD patients compared to controls. Immunoprecipitation, a third type of assay using conformational antibodies, achieved a selective pull-down of A β oligomers from AD brain extracts [37] or the "protease resistant core" (A β 20-42) of A β oligomers in human and tg mouse brain extracts [71]. However, further use of this antibody as a diagnostic tool is minimal since it does not recognize A β in cerebral spinal fluid (CSF), plasma and amyloid deposits *in vivo*.

Neuropathology. Assessment of conformation-specific antibodies commonly includes neuropathological examination of Tg-mice and human brain tissue. Prior to these antibodies, early research typically labeled amyloid fibril plaques with the beta-pleated sheet markers Congo red and thioflavin S. With these probes, plaque load was found not to reflect the severity of the disease [25, 79, 80]. This traditional labeling failed to detect a significant portion of the A β -derived toxins that are present. Conformation-specific antibodies, on the other hand, detect and localize small oligomeric species now implicated in pathogenesis [35, 81-83]. The antibodies thus provide a rationale for the disconnect between disease severity and plaque

load that has confounded development of effective AD treatments.

As previously mentioned, distribution is distinct from fibrillar amyloid deposits detected by thioflavin-S staining, a pattern first observed by Glabe and colleagues using the oligomer-specific antibodies generated at their lab [52]. These results establish the *in situ* presence of oligomers distinct from fibrils, and they validate the conclusion obtained from immunoblot assays that A β oligomers accumulate in AD brain. Conformational antibodies are also characterized by their ability to detect specific A β species in brain tissue. Some antibodies label diffuse plaques (putatively soluble A β oligomers) without detecting dense senile plaques (A β fibrils) [52, 55], while many detect all aggregated A β species [31, 57, 58, 62, 63]. Still others have been shown to recognize only fibrillar A β , detecting senile plaques without labeling diffuse plaques [60]. A unique example of the use of conformational antibodies to detect specific A β species is the discovery of a new form of amyloid in reactive astrocytes of AD brain. Using NU-6, an aggregate-specific monoclonal developed by Klein group [57], immunoreactivity was found in cells of frontal cortex sections that presented morphology compatible with reactive astrocytes. Labeling of the entire astrocyte (cell body and processes) was evident in severe AD cases whereas in mild AD only the cell body was labeled; no labeling was seen in nondemented controls, nor were amyloid plaques labeled in any section. Double-labeling brain sections from a severe AD case with NU-6 and a polyclonal antibody against glial fibrillary acidic protein confirmed that the NU-6 labeling was astrocytic. Characterization of the astrocytic amyloid is ongoing. Taken together, results confirm that conformational antibodies are useful tools for distinguishing between the various A β pathologies in human and/or Tg-AD mice models.

In the long term, conformational antibodies offer a unique perspective for understanding the relationship between the presence of the A β toxin and dementia. Most studies extract toxins from the brain, measure their abundance, and assess relationship with cognitive scores. What is more relevant is the distribution of these toxins relative to existing brain circuits, where one wants to follow the presence of the toxin in specific synapses rele-

vant to particular behaviors. Only antibodies that target a specific toxin have the necessary precision.

In addition to determining broad-scale distribution of oligomeric proteins in AD brain, conformation-specific antibodies have been used to detail cellular-level localization at early stages of the disease. The salient question is where oligomers first accumulate, which has implications for mechanisms of toxicity. Evidence with ADDL-generated antibodies shows early stage AD brain sections with immunoreactivity selectively surrounding cell bodies [57, 77]. This pattern, which develops before the onset of other major pathology, is reminiscent of the diffuse synaptic-type deposit observed in prion-associated diseases [84, 85]. Consistent with these observations, other conformational antibodies applied to AD brain sections have indicated oligomer localization to dendritic arbors [77]. Conformation-specific antibodies have been used further to distinguish between intracellular A β build-up and extracellular deposition [55, 66, 86, 87]. Investigation of Tg-mouse brain sections indicates an earlier robust build-up of oligomers within neurons than is evident in human tissue, potentially due to a more excessive production of A β in the animal models [86, 87].

Cellular Mechanisms: Targeting Synapses.

Conformational antibodies have been used to investigate how ADDLs interact with cells, the pathological consequences, and the underlying mechanisms. A prominent finding is that ADDLs act as pathogenic ligands, binding with specificity to only a subpopulation of neurons. This binding is readily detected by conformation-specific antibodies, and it enables characterization of changes localized to the targeted cell.

In experiments with cultured hippocampal cells, a widely used model for synaptic mechanisms, ADDL binding shows a striking coincidence with PSD-95 puncta ($93 \pm 2\%$ colocalization)[77]. In this model, essentially all clusters of PSD-95, which is a prominent scaffolding protein involved in signal transduction, occur at synapses [88]. ADDL binding sites also were juxtaposed to synaptophysin-positive presynaptic terminals, although complete coincidence of ADDL and synaptophysin immunoreactivities was uncommon.

ADDL binding sites thus were almost completely localized to synapses [77]. Most significantly, an identical pattern of synaptic binding was obtained with ADDLs in extracts of AD brain. Conformational antibodies have demonstrated that synaptic binding by brain and synthetic A β oligomers is associated with ligands > 50kDa in size [36, 69, 77, 89].

Synapses targeted by ADDLs comprised approximately half of those that were present, consistent with specific ligand behavior. ADDL binding sites localized to synaptic spines and overlapped with NMDA receptor (NR1) immunoreactivity, consistent with the association of PSD-95 and NMDA glutamate receptors in excitatory hippocampal signaling pathways [90]. Synaptic localization indicated by microscopy was confirmed and extended by experiments in which synaptosomes were incubated with ADDLs and subjected to magnetic bead immunoisolation using ADDL-selective antibodies. Detergent extraction of synaptosomes yielded an ADDL binding complex still capable of pulldown by immunobeads. The complex was identified as postsynaptic because syntaxin, a presynaptic active-zone protein, remained in the unbound fraction. Results from microscopy and biochemical fractionation using conformational antibodies thus are in harmony with the conclusion that ADDLs bind to excitatory synapses at postsynaptic sites. This is consistent with their putative ability to locally initiate synaptic dysfunction and memory failure.

Double-labeling experiments revealed that ADDL-targeted neurons undergo pathological changes characteristic of AD, manifesting oxidative stress and AD-like tau hyperphosphorylation [91, 92]. Synaptic receptors essential for plasticity and memory were found to be down-regulated (NMDA glutamatergic and insulin receptors [93, 94]). Loss of insulin receptors, moreover, provided a putative mechanism to explain the insulin resistance apparent in AD brain [94]. The cytoskeleton of spines, marked by antibodies to the F-actin binding protein drebrin, revealed dramatic changes in spine morphology [93]. By 6 h, dendrites showed abnormally elongated protrusions rather than those characteristic of mature dendritic spines. By 24 h, spine density on the ADDL-targeted neurons had decreased more than 50%.

The change in spine appearance caused by ADDLs is especially interesting because the elongated shape resembles that of immature spines or of diseased spines found in mental retardation and prionoses [95], while synapse loss is the best known correlate of AD dementia [79]. All findings to date using conformational antibodies are consistent with the possibility that ADDL toxicity provides a unifying pathogenic mechanism for AD, explaining why the early disease is specific for memory and also accounting for major features of AD neuropathology.

Pathogenic Structure. Results using conformational antibodies have shown that the toxic species are not monomers, and most likely not fibrils, but rather smaller soluble A β oligomers [30, 36, 52, 60, 63, 66, 67, 69, 70, 78, 96, 97]. There is no consensus, however, regarding which oligomeric A β species contribute most significantly to AD pathogenesis. Dimers, trimers, tetramers, 12mer/A β *56, globulomers, and protofibrils are being examined for disease relevance. Lack of consensus is likely influenced by factors that bias results, such as the presence of detergents, pH, temperature, and methodology employed [98-102], including the specificity of the antibodies used during investigations. It is intriguing that, despite an overlap in size, aggregated A β species can vary in toxicity. For example, toxic and non-toxic oligomers with identical molecular mass have been differentiated by conformational antibodies that only bind to the toxic species [70, 77, 96]. These results suggest that divergent pathways exist for oligomerization of A β , with some pathways leading to toxicity and others producing benign structures. Extending this concept, at the other end of the spectrum, the development of several antibodies [52, 70, 103] that recognize the oligomeric structures of many amyloidogenic proteins and peptides (e.g. A β , α -synuclein, IAPP, etc.) has led to the idea that toxins in multiple proteinopathies express a common tertiary structure.

Drug Discovery. The ability of conformational antibodies to measure small quantities of toxic oligomers in solutions containing monomeric precursors makes them useful tools for drug discovery, and is of value for identifying small molecules capable of blocking oligomer formation. For example, in a sensitive immunoassay using conforma-

tional antibodies, Chang *et al.* [76, 96] were able to follow the time course of ADDL assembly from a starting solution containing 10 nM A β , showing assembly occurs even at such low doses within minutes. Using this assay, a specific fraction, EGb, from *G. biloba* was found to completely block ADDL assembly at 1.0 μ g/mL, in harmony with its reported neuroprotection [96, 104]. Previous studies had attributed the neuroprotective effects of EGb to putative antioxidant properties [105], but these findings indicated an additional capacity for blocking ADDL formation. Dotblot immunoassays also have been used to screen small libraries to define molecular structures active against the assembly of A β into ADDLs [68, 76, 106] and to identify substituted aromatic compounds that effectively block ADDL assembly and neurotoxicity [68, 76, 106, 107]. In addition to direct application in discovery of novel compounds, use of conformation-selective antibodies offers the potential to monitor ADDL levels in clinical trials, thereby offering a means to accelerate drug development by providing molecular readouts of efficacy.

Diagnostic Assays. The selectivity and increased antigenic affinity of conformational antibodies offer an excellent platform for development of AD diagnostics. They overcome major problems with current assays that include the extensive overlap between diseased and control patients and the very low concentrations of soluble pathogenic markers in biological fluids (e.g. CSF or plasma). One example of a preliminary diagnostic assay based on ADDL-generated conformational antibodies is the biobarcode [73]. In this assay, magnetic microparticles and gold nanoparticles conjugated with biobarcode DNA are functionalized with anti-ADDL antibodies. These particles are used to measure ADDL concentrations. Multiple copies of the DNA barcode amplify the signal allowing extremely low A β oligomer concentrations to be quantified. Results show A β oligomer levels in CSF from subjects diagnosed with AD were markedly and consistently higher than levels in CSF from age-matched control subjects. This assay demonstrates a sensitivity improvement over previously used methods with a limit of detection in the range of \sim 100aM. A second assay [74] that monitors the interaction between conformation sensitive antibodies and A β oligomers uses a local-

ized surface plasmon resonance (LSPR) nanosensor. Measurements of LSPR spectral shifts from AD and control CSF samples showed a 4-fold higher shift in AD over control, verifying that this assay has the potential to distinguish extremely small oligomer concentration differences.

Immunotherapy. In cell biology experiments, conformational antibodies effectively protect neurons from A β oligomeric toxins. Oligomer-specific antibodies block the attachment of synthetic and human brain-derived oligomers to nerve cell surfaces and thus prevent oligomer-induced pathological responses [31, 58, 63, 66, 67, 71, 77, 92, 93, 108] and associated toxic neuronal cell cycle events [109]. Some antibodies have been shown to inhibit oligomerization or mediate A β fibril disaggregation [63, 66, 67, 110]. Oddly, one such antibody induces fibril disaggregation, but has no effect on pre-formed A β oligomers [63]. These studies demonstrate the potential of conformational antibodies to serve as effective neuroprotectants by preventing either toxin binding or toxin formation. Additional mechanisms *in vivo* appear to include the equilibrium shift of A β assembly to monomeric forms, promoting clearance, and the induction of toxin clearance mediated by scavenger cells [111].

Both active and passive immunization strategies have been proposed for clearing A β from the brains of Tg-AD mice and AD patients, but immunotherapy development has largely relied on antibodies that are not necessarily conformational [112] (for a comprehensive review, see [111]). Success with active immunization of animal models by A β peptide injections [4, 6, 7, 113] or nasal administration [114, 115] has been shown to reduce plaque burden and improve cognition in Tg-mice. However, a small number of preclinical studies have successfully tested immunization with conformational antibodies by using intraperitoneal or intracerebral injection [60, 62, 63, 71, 72]. Results include a rapid improvement in spatial learning and memory [60], reduction in A β deposits in Tg-AD mouse brains [62, 63, 72], inhibition of A β 42 assembly into fibrils [63], mediation of A β 42 fibril disaggregation [63], and protection of neuronal cells from A β 42 oligomer and fibril mediated toxicity [63]. Strikingly, cognitive improvement in these mice models does not neces-

sarily correlate with a reduction in plaque load. A likely explanation for the phenomenon is that these therapeutic antibodies immunoneutralize small, soluble oligomers of A β that have been implicated in AD synapse failure [30, 47]. The antibodies also have been used to show that A β oligomers exist in a dynamic relationship between intracellular and extracellular pools within the brain [86]. The effects of conformational antibodies appear to be longer-lasting and more efficient than conventional A β antibodies in reversing as well as preventing early synaptic deficits in Tg-AD mice [60, 116]. These oligomer-specific antibodies also were found to reverse tau and plaque pathology [117]. Most interestingly, conformational antibodies were found to be effective in restoring cognitive behavior [60].

Translation of findings from animal model immunotherapy to human trials occurred rapidly, but, while active immunizations proved beneficial in Tg-AD mouse models, severe complications arose in human subjects, causing the trials to be halted and raising questions about the safety of active vaccination [5, 118, 119]. Of note, ~80% of the immunized subjects failed to develop anti-A β antibody titers, indicating that the A β self-antigen is not a strong immunogen in the elderly and suggesting that alternative immunotherapeutic strategies should be pursued. Nonetheless, in individu-

als that developed an immune response there were significant indications of cognitive benefits. Passive vaccines are under development to avoid the poor responses and side-effects linked to active vaccination protocols [111]. In a novel approach, Dodel and colleagues as well as Relkin and colleagues have investigated intravenous human immunoglobulin as a therapeutic agent based on the presence of spontaneously-formed antibodies targeting A β species. Clinical trials have provided indications of cognitive benefits [120, 121]. Results from cell culture experiments have shown the preparation can neutralize the toxicity of oligomeric A β [122]. A potential concern is that antibodies may produce microhemorrhages by attaching to amyloid surrounding cerebral blood vessels. This may be less of a problem if vessel plaques are largely made up of A β 40 and antibodies can be made selective for A β 42-derived oligomers. Taken together, the preclinical and clinical data suggest that the most effective therapeutic approach for the treatment of AD, and potentially other proteinopathies, ultimately will involve the use of conformation specific antibodies.

CONCLUSION

Pathogenic events that initiate memory loss, the major manifestation of early AD, are induced by the accumulation of potent neurotoxins that com-

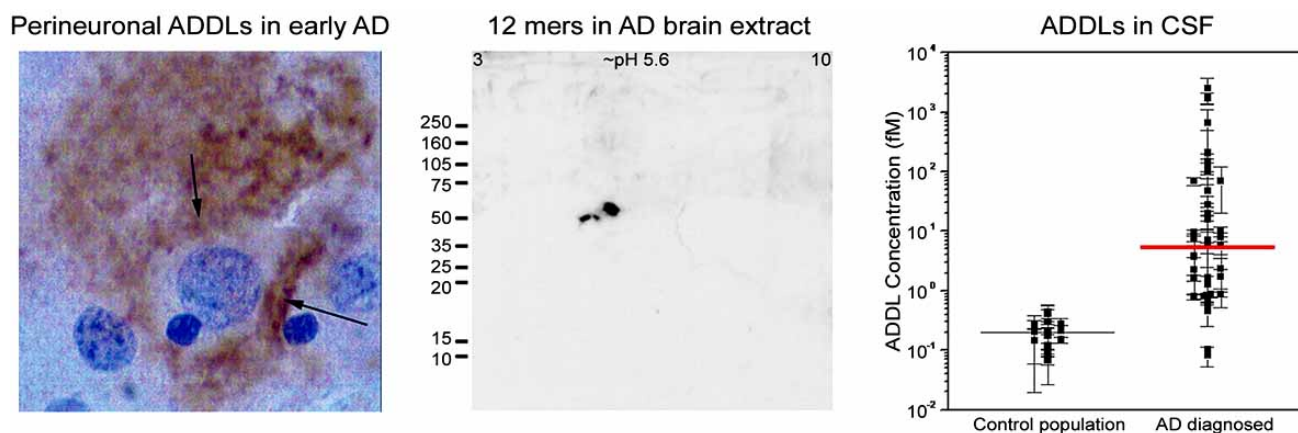


Fig. (1). Three applications of conformation-specific antibodies. *Left* Immunohistochemistry of brain section from early stage AD patient labeled with ADDL-selective polyclonal antibody. Perineuronal staining of dendritic arbor is evident before other facets of neuropathology emerge (from [77]); *Middle* Western blot of soluble extract of AD brain subjected to two dimensional electrophoresis. ADDL-selective antibody identifies a prominent 12 mer (54 kDa), also present in synthetic ADDL preparations but not in control brain extracts (from [69]); *Right* Ultrasensitive nanotechnology-based sandwich immunoassay with polyclonal and monoclonal conformation-specific antibodies showing detection of ADDLs in human CSF has promise for future diagnostics (from [73]).

prise conformationally unique A β species. To study these toxic A β species, antibodies have been developed that prevent binding of aggregated A β and the resulting responses in cultured cells. Furthermore, these antibodies have been successful at improving memory dysfunction in Tg mice.

Multiple techniques have been employed for generation of conformational antibodies. Most use specific A β structures or modified peptide constructs as the immunogen, although library screening has also been used to identify antibodies. Antibodies have been produced that can identify specific forms of A β oligomers, protofibrils, or fibrils, while others recognize a common structural epitope within amyloidogenic proteins regardless of sequence specificity.

Conformationally sensitive antibodies are providing a wealth of new information about A β structure and function in AD. These antibodies are being employed in studies of A β toxin assembly mechanisms, providing valuable information about which A β species to monitor for diagnostic assays and what forms of assembled A β to target for immunotherapy. Antibodies with a conformational epitope have localized oligomers, protofibrils, and fibrils in brain slices from Tg mouse models and humans. The passive immunization of Tg-AD mice with conformational antibodies has shown a reduction of disease-related phosphorylated tau and the elimination of plaques, emphasizing the importance of conformation-specific antibodies in AD immunotherapy development. Lastly, conformational antibodies have been used in the development of extremely sensitive diagnostic assays, a tool that is currently lacking and could expedite treatment for those beginning to suffer from the disease. Three examples illustrating the use of conformationally-sensitive polyclonal and monoclonal antibodies are found in Fig. (1), which shows the dendritic localization of oligomeric toxins in early AD, the biochemical analysis of AD brain-derived oligomers, and a prototype diagnostic assay for the disease-dependent accumulation of oligomers in CSF.

One relevant question is how soon before these antibodies are available to patients and the general research community? Several Phase I clinical trials are in progress that use immunotherapy protocols.

In general, the available public information about the various A β antibodies used for passive vaccination specifies little about the nature of the antibody or its epitope. Likewise, active vaccination strategies in this category have little public information about the form of the A β antigen, other than that at least one is designed to minimize inflammatory responses. However, the epitopes of two antibodies in Phase II or Phase III clinical trials, respectively, have been characterized. Neither LY2062430, which is a humanized monoclonal antibody directed against an A β central domain, and Bapineuzomab, also a humanized monoclonal but one that is directed against the N-terminus of A β , appear to be against a conformational epitope [111]. Thus, the advent of immunotherapy with conformationally directed vaccines is still in the fundamental research stage.

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CONFLICT OF INTEREST

WLK is co-founder of Acumen Pharmaceuticals, licensed by Northwestern University to develop therapeutics and diagnostics targeting ADDLs.

Abbreviations

A β	= Amyloid beta
AD	= Alzheimer's disease
ADDLs	= Amyloid beta-derived diffusible ligands
CSF	= Cerebral spinal fluid
Tg	= Transgenic
IF	= Immunofluorescence
WB	= Western blot
IHC	= Immunohistochemistry
IP	= Immunoprecipitation
IEM	= Immuno-electron microscopy
IAPP	= Islet amyloid polypeptide

ROS = Reactive oxygen species

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