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Linear and conformation specific antibodies in aged beagles after prolonged vaccination with aggregated Abeta

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ABSTRACT

Previously we showed that anti-Aβ peptide immunotherapy significantly attenuated Alzheimer's-like amyloid deposition in the central nervous system of aged canines. In this report we have characterized the changes that occurred in the humoral immune response over 2.4 years in canines immunized repeatedly with aggregated $A\beta_{1-42}$ (AN1792) formulated in alum adjuvant. We observed a rapid and robust induction of anti-Aβ antibody titers, which were associated with an anti-inflammatory T helper type 2 (Th2) response. The initial antibody response was against dominant linear epitope at the N-terminus region of the $A\beta_{1-42}$ peptide, which is identical to the one in humans and vervet monkeys. After multiple immunizations the antibody response drifted toward the elevation of antibodies that recognized conformational epitopes of assembled forms of Aβ and other types of amyloid. Our findings indicate that prolonged immunization results in distinctive temporal changes in antibody profiles, which may be important for other experimental and clinical settings.

Introduction

Alzheimer's disease (AD) is associated with progressive cognitive decline, neuronal loss and the accumulation of senile plaques and neurofibrillary tangles in affected regions of the brain (Braak and Braak, 1991; Mirra et al., 1991). The amyloid beta peptide (Aβ) is a major component of senile plaques, and the original amyloid cascade hypothesis (Hardy and Higgins, 1992; Selkoe, 1996) proposed that the accumulation of amyloid plaques was the principal factor in AD pathogenesis, however the cascade hypothesis has evolved to include neurotoxic small soluble Aβ aggregates or oligomers (Golde et al., 2006; Lesne et al., 2006; Lue et al., 1999; Mucke et al., 2000; Walsh et al., 2002a; Walsh et al., 2002b). Thus, therapeutic interventions currently being tested are targeted towards slowing production, accumulation or increasing clearance of pathological Aβ species (Selkoe, 2007; Selkoe and Schenk, 2003).

Based on the success of immunotherapy in transgenic mice (Janus et al., 2000; Morgan et al., 2000; Petrushina et al., 2007; Schenk et al., 1999; Seabrook et al., 2007), a clinical trial was initiated in AD patients who were immunized with aggregated Aβ42 (AN1792) formulated in QS-21 adjuvant. However, a subset of patients (6%) developed aseptic meningoencephalitis (Orgogozo et al., 2003; Schenk, 2002), and the clinical trial was halted with patients receiving only 1–3 injections of AN1792 instead of 6 proposed in the Phase IIa protocol. Evaluation of the complete study cohort revealed limited cognitive benefits and "less worsening" of clinical outcome measures (Gilman et al., 2005). In spite of the failure of AN1792 there is still considerable interest in active and passive immunotherapeutic approaches for AD (http://www.clinicaltrials.gov/).

Aged dogs show a decline in learning and memory, which correlates with a progressive increase in Aβ pathology in the brain (Cummings et al., 1996; Head et al., 1998, 2000; Milgram et al., 2002, 1994; Selkoe, 1996). We recently reported that immunizing aged beagles with aggregated $A\beta_{1-42}$ was associated with a significant reduction in brain Aβ (Head et al., 2008), however, there were only minor improvements in learning and memory observed. Similar outcomes have now been confirmed in a recent study of 8 AD patients in the AN1792 trial who have come to autopsy. These patients showed significantly less Aβ plaque deposition, however, none of the immunized patients showed any slowing of dementia and all eventually progressed to end stage disease (Holmes et al., 2008).

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In this report we describe changes in the humoral response in individual dogs after immunization with $A\beta_{1-42}$ over 2.4 years. We observed that the initial antibody response was primarily against linear epitopes, however after multiple immunizations the antibody response drifted toward antibodies that recognized conformational epitopes of Aβ, as well as other types of amyloid. This is the first report on the effects of long-term active immunization with $A\beta_{1-42}$ peptide on the humoral immune response in a large animal model of Aβ-pathogenesis, and the results provide new insights into changes that occur in response to repeated immunization with the full-length peptide.

Materials and methods

Animals

The longitudinal study included 20 beagles (Head et al., 2008). Briefly, the ages of the animals ranged from 8.4 to 12.4 years at the beginning of the study (18 females and 2 males). All animals were thoroughly examined prior to inclusion in the study and were determined to be in good health. On the basis of baseline cognitive test results, 9 animals were assigned to be immunized with Aβ, 6 received the adjuvant alum only and 5 received saline only injections. As we reported earlier (Head et al., 2008), in the first year of study one saline injected dog was removed from the study because of an oronasal fistula. A second animal developed blindness (aggregated Aβ-immunized group) and was maintained on the study but could not complete the cognitive testing protocol. A third animal in alum control group was removed in the second year of the treatment because of mammary carcinoma that spread into the lymphatic system. In last year of the study, a fourth animal from Aβ-immunized group was removed after poor recovery from a CSF tap. However, the treatment overall was well tolerated by the animals and no symptoms of adverse events were noted. All procedures were conducted in accordance with IACUC approved animal protocols and the NIH Policy on Humane Care and Use of Laboratory Animals.

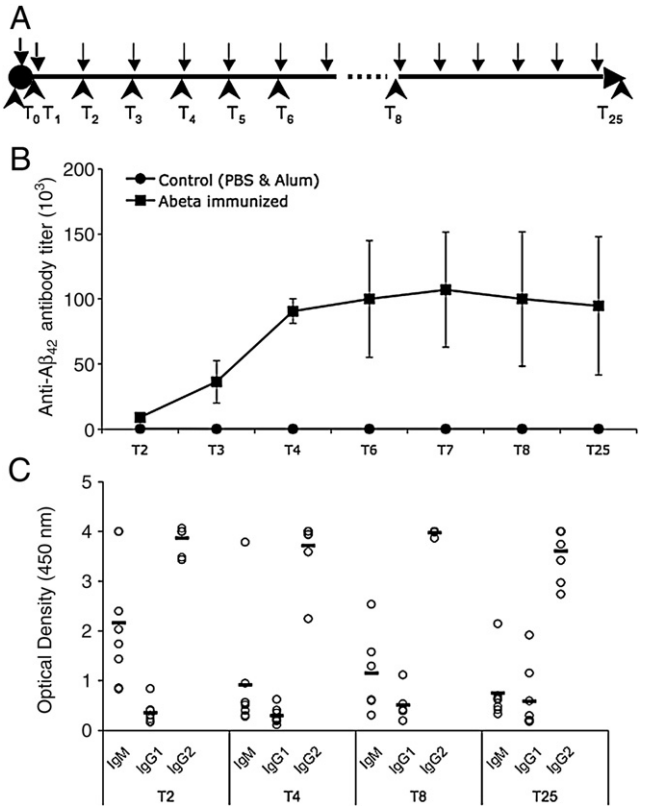

Fig. 1. Immunization of aged canines with aggregated $A\beta_{1-42}$ induces a robust and relatively uniform antibody response. (A) Time line of injections (black arrows) and blood collection (black arrowheads) in older canines. (B) Anti-Aβ antibody levels detected with ELISA reached a plateau after 4 injections and were maintained until the end of the study. (C) Anti-Aβ antibodies induced by vaccination were primarily of the IgG2 (anti-inflammatory) isotype. Open circles represent the data for individual animals, lanes are the value of a mean. (T = blood collection time points). Bars represent means, and error bars represent SEM.

Immunization

Aggregated $A\beta_{1-42}$ immunogen was prepared as previously described (Head et al., 2008). Briefly, $A\beta_{1-42}$ was prepared by adding 1 ml of phosphate buffered saline (PBS pH 7.5) to 1 mg of peptide, and incubated overnight at 37 °C in a water bath with moderate shaking prior to conjugation with the adjuvant. To prepare Aβ for immunization, 0.5 mg of aggregated Aβ suspension was mixed with 50 µl of 2% aluminum hydroxide (Alum, Accurate Chemical, Westbury, NY) and 450 µl of PBS. Control animals either received alum only in saline ($n=6$) or saline only ($n=5$). Animals were immunized subcutaneously in the back of the neck and monitored for adverse reactions. After 2 weeks, animals were boosted with an additional injection. Following the first 2 injections, animals received a single injection each month for a period of 2.4 years. Blood from experimental animals was collected prior to the start of the study (pre-bleed) and immediately prior to each vaccination monthly for the first 6 months and every 6 months after that (Fig. 1A). Blood was collected in 10 cm^3 red top tubes, centrifuged and the supernatant (serum) was used for the anti-Aβ antibody analyses.

Anti-Aβ antibody titers and linear epitope mapping

The titers of anti-Aβ antibodies were measured by ELISA as previously described (Cribbs et al., 2003; Head et al., 2008). Briefly, 96-well plates (Immulon 2HB, Thermo Fisher Scientific, Waltham, MA) were coated with 2.5 µM of $A\beta_{1-42}$ protein in carbonate coating buffer (CCB) pH 9.6 (Sigma-Aldrich) and incubated overnight at 4 °C. The wells were washed and blocked with 3% non-fat dry milk for 1 h at 37 °C with shaking. After washing, serial dilutions of all serum samples were added to the wells and the plates were incubated for 1 h at 37 °C with shaking. After washing, HRP-conjugated rabbit anti-dog IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were added at 1:2000 dilution for 1 h at 37 °C with shaking, wells were washed, and Ultra-TMB ELISA substrate (Pierce, Rockford, IL) was added to develop the reaction for 15 min. The reaction was stopped by adding 2 N H_2SO_4 and the plates were analyzed on a Synergy HT Spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 450 nm. The serum end-point titer was defined as the maximal serum dilution in which the OD for the antibodies was 3 times higher than the OD values of the blank wells. To calculate anti-Aβ antibody concentration, we used the mouse monoclonal 6E10 antibody (Covance, Princeton, New Jersey) as a standard and calculated dog antibody concentrations using automatic KD4 software (Bio-Tek Instruments). B-cell linear epitope mapping was performed as described previously (Cribbs et al., 2003). Briefly, small overlapping 15- and 17-mer peptides (Invitrogen) spanning the $A\beta_{1-42}$ peptide sequence as shown in Fig. 2 were coated on ELISA plates as above and individual dog sera from different time points were serially diluted and added to the wells. Serum binding was detected with HRP-conjugated rabbit anti-dog antibodies, followed by Ultra-TMB ELISA substrate as above.

Anti-Aβ antibody isotype determination

To determine the specific isotypes of induced anti-Aβ antibodies generated by immunized dogs we followed the protocol established