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Antibody response and plasma A β 1-40 levels in young *Microcebus murinus* primates immunized with A β 1-42 and its derivatives

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Abstract

We have been developing A β derivative vaccines with the objective to improve the safety of A β targeting immunotherapy. Our A β homologs are designed to have less direct toxicity and to produce a modified immune response compared to A β . In extensive mouse studies, all our vaccines have improved cognition in transgenic mice while eliciting different immune responses and reducing brain amyloid burden to a variable degree. While we are continuing to characterize these vaccines in mice, in preparation for studies in old primates and for human trials we assessed their effect in young lemur primates ($n = 25$) that with age develop A β plaques and tau aggregates as seen in Alzheimer's disease.

In the primates, all the peptides administered with alum adjuvant elicited a moderate to robust anti-A β IgM response. A β 1-42, K6A β 1-30 and K6A β 1-30[E₁₈E₁₉] resulted in a high anti-A β IgG response, whereas A β 1-30[E₁₈E₁₉] produced a weaker more variable IgG titer. Notably, 22 weeks after the 3rd immunization, IgM and IgG levels in derivative-vaccinated primates were similar to preimmune values whereas A β 1-42 treated primates maintained a moderate IgG titer. The increase in antibodies that recognized A β 1-40 often correlated with increase in A β 1-40 in plasma, which suggests that the antibodies were binding to A β in vivo. Interestingly, significant transient weight gain was observed (K6A β 1-30-, A β 1-30[E₁₈E₁₉]- and A β 1-42-treated) or a trend in the same

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direction (K6A β 1-30[E₁₈E₁₉]-treated, adjuvant controls) following the injections. Based on these findings, we have chosen K6A β 1-30 for immunizations in old primates as the antibody response to this vaccine was less variable compared to other A β derivatives. Our present findings indicate that most of our A β derivatives elicit a substantial antibody response in primates, and importantly this effect is reversible which enhances the safety profile of our approach.

Keywords

Amyloid- β ; *Microcebus murinus*; Lemur; Primate; Immunization; Alum

1. Introduction

Prior to the occurrence of brain inflammation in the Elan/Wyeth AN-1792 trial, we raised concerns about administering full-length A β 1-42 self-antigen in humans, and we advocated the use of both adjuvants and immunogens that favored a Th2 response promoting antibody production instead of a Th1 response which mediates a cytotoxic T-cell response [1]. The primary objective in designing our A β derivatives was to maintain antibody epitopes while reducing their β -sheet content compared to A β 1-42, in order to eliminate direct toxicity and amyloid seeding potential. These modifications also altered or removed potential T-cell epitopes as determined by computer algorithms [2,3]. Most of the potential T-cell epitopes in A β 1-42 are located in the C-terminus of the peptide (which we removed) or in the hydrophobic region in the middle of the peptide (that we modified). Interestingly, recent findings in the prion field also indicate that immune responses to α -helical structures appear to preferentially involve the Th2 pathway whereas a β -sheet conformation favors Th1 activation [4].

Our initial report was on K6A β 1-30 which has 6 lysines on the N-terminus to increase immunogenicity and enhance solubility. This modification in addition to removal of the C-terminal amino acids of A β 1-42 also reduced its propensity to form β -sheets. The main antibody epitopes are located within the first 30 amino acids of the peptide and this peptide as expected elicited a similar albeit somewhat less antibody response as A β 1-42 in mice which resulted in a comparable therapeutic efficacy [1]. Concurrently, we have been determining the therapeutic potential of other A β derivatives with internal substitutions to further diminish T-cell response [5]. It appears that a robust immune response towards A β is not needed to improve cognition since some of our A β derivatives elicit only very modest anti-A β antibody titers, while producing cognitive improvements [5–7]. Interestingly, one of our A β derived immunogen, K6A β 1-30[E₁₈E₁₉], primarily produced a T-cell independent IgM response, and the anti-A β IgM titer correlated inversely with brain plaque burden [5]. IgM does not cross the blood brain barrier because of its size [8,9]; hence our observations indicate that it may be sufficient to clear A β peripherally to reduce amyloid burden in the brain, as we and others have previously suggested [1,10]. One of the advantages of this approach is that the potential of antibody-mediated CNS inflammation is avoided. Also, because of its T-cell independence, the IgM response is reversible and it subsides within a few weeks of vaccination. This reversibility is important if any adverse reactions occur in the course of the vaccination. In other words, this type of response resembles passive immunization but avoids the possibility of an anti-idiotypic response and subsequent side effects of multiple antibody injections as we have discussed previously [11,12].

The different immunogenicity profiles of these A β derivatives in transgenic models are intriguing but prior to clinical trials it is necessary to assess potential AD immunotherapy in other models. Ideally, those models should have an immune system that is closer to humans than the mouse equivalent; and endogenous A β levels that are comparable to humans. Non-human primates are the obvious choice and many of the subspecies develop similar plaque

pathology as seen in AD. Two preliminary findings in four Vervet-[13] and two Rhesus primates [14] show that these animals like humans develop an immune response to A β 1-40/42 that may have resulted in amyloid clearance [13]. Furthermore, immunization of aged canines with fibrillar A β 1-42 reduces A β 1-40/42 levels as well as their diffuse A β plaques, which is associated with stabilization of prefrontal-dependent learning ability [15]. The advantage of the *Microcebus murinus* lemur primate, which we have chosen to use, is that these animals develop tau pathology in addition to amyloid deposition, and are therefore better models of AD than many other primates [16–18]. They can, therefore, also be used to assess experimental therapy targeting pathological tau such as we and others have recently described in mouse models [19–21]. Also, their small size reduces housing cost and facilitates experimental work. This allows larger number of animals to be included in each experimental group, increasing the reliability of the data.

We report that immunization with A β derivatives in *M. murinus* can result in different antibody response than in mouse models. Importantly, the reversibility of this effect renders it a safer approach for future human trials.

2. Materials and methods

2.1. Peptides

Synthesized by the solid-phase technique (Keck, Yale Univ.), as we have described previously [1,5,7].

2.2. Primates

M. murinus (mouse lemur primate) develops A β plaques and hyperphosphorylated tau aggregates with age [16–18]. The advantages of these primates over simians are: (1) Smaller size (70–150 g) and shorter lifespan (10–13 years [16]), (2) Easier reproduction, (3) Both A β and tau pathology develop with age whereas simians only develop A β pathology. The animals were randomly obtained from a colony of 250 primates kept in our zoological facility in Montpellier, France. The primates employed were disease-free and had never been subjected to any experimental treatment. Twenty-five animals were enrolled in the study (five per group: Table 1).

2.3. Injections and bleeds

The time line for injections and bleeds is depicted in Fig. 1. Five animals per group received a subcutaneous injection with A β 1-42 or its derivatives in alum adjuvants (Brenntag Biosector, Denmark). The peptides were mixed with alum adjuvant at a concentration of 1 mg/mL and the solution was rotated overnight at 4 °C prior to administration to allow the peptide to adsorb onto the aluminium particles. Controls received alum adjuvant alone ($n = 5$ per group). The primates received the second and third injections at 2 and 6 weeks. The fourth, fifth and sixth injections were at 42, 44 and 48 weeks, respectively. The primates were bled prior to the first immunization (T0), 1 week following the second (T1, 3 weeks) and third injection (T2, 7 weeks). T3 was at 28 weeks (22 weeks following the third injection) to assess the reversibility of the immune response. T4 and T5 were performed at 45 and 49 weeks, respectively (1 week following the fifth and sixth injection).

2.4. Antibody levels

Determined at 1:200 dilution of plasma using an ELISA assay as we have described previously [1,5], where the immunogen or the full-length A β 1-40/42 peptide was coated onto microtiter wells (Immulon 2 HB, ThermoScientific, Waltham, MA). Antibody levels were detected by

an anti-primate IgG and IgM linked to a horseradish peroxidase (Alpha Diagnostics, San Antonio, TX).

2.5. A β 1-40 levels in plasma

For measurements of free A β 1-40 in plasma, 10% dilution of untreated plasma were used, and the detection was performed by an ELISA kit (Biosource, Camarillo, CA) as described by the manufacturer. Under these conditions, A β 1-42 levels in plasma were below the limit of detection.

2.6. Staining of AD brain tissue

Paraffin embedded brain sections (10 μ m) through the superior frontal/cingulate gyrus from a familial AD case were stained with plasma dilutions (1:100) from several primates at different bleeds with varying A β antibody levels using standard protocol as described previously in more detail [22]. The donor met the NIA Reagen/CERAD criteria for definitive AD, with a Braak and Braak stage VI/VI, and the tissue was obtained from the NYU ADCC Neuropathology Core brain bank (grant AG 08051). The sections were deparaffinized and hydrated through xylene and ethanol gradient and were then washed in water followed by overnight incubation at 4 °C in plasma (1:100) or monoclonal antibody dilutions (6E10/4G8 (courtesy of Richard Kascak, IBRDD, Staten Island), 1:1000). The plasma samples were diluted in a blocking diluent as described previously [23] that contained 2% bovine serum albumin and 10% goat serum to prevent non-specific staining. MOM diluent (Vector Labs, Burlingame, VT) was used for the 6E10/4G8 dilution. After three washes in PBS containing 0.1% Triton X-100 (PBS-T) for 10 min each, sections were incubated for 1 h in biotinylated goat anti-monkey IgG (1:200; Alpha Diagnostics) and biotinylated horse anti-mouse IgG (1:200; MOM kit). Subsequently, sections were washed again three times in PBS-T followed by 1 h incubation in avidin-peroxidase complex (1:200 dilution; Vector Labs). The sections were then washed in 0.2 M sodium acetate buffer and reacted with diaminobenzidine tetrahydrochloride (0.35 mg/ml; Sigma) with nickel intensification (nickel ammonium sulfate 25 mg/ml; Fisher Scientific, Fair Lawn, NJ) in the same buffer containing 0.3% hydrogen peroxide (Sigma, St. Louis, MO).

2.7. Statistical analysis

All the data were analyzed with GraphPad Prism 4.3 (San Diego, CA) or Statistica 6 (Statsoft Inc. Tulsa, OK). Correlation between plasma A β 1-40 levels and anti-A β 1-40 IgG or IgM response was analyzed by Pearson *r* correlation or Spearman rank correlation if the data failed at least two of three normality tests (Kolmogorov–Smirnov, D'Agostino and Pearson omnibus, and Shapiro–Wilk normality tests). The weight of the primates at different time points within each group was analyzed by one-way ANOVA repeated measures and Dunnett's post hoc test in which the preimmunization weight was compared to the weight of the animals at subsequent intervals.

3. Results

Very low levels of IgG and IgM antibodies that recognized A β 1-40 or A β 1-42 were observed in control primates that received adjuvant alone (Fig. 2A and B). We have previously observed similar degree of autoantibodies against A β in mouse models [1,5,7]. Of the three A β derivatives, the most consistent antibody response was observed with K6A β 1-30 (Fig. 2C and D) but a similar degree of response was observed with K6A β 1-30[E₁₈E₁₉] albeit more variable (Fig. 2E and F). A β 1-30[E₁₈E₁₉] elicited the lowest antibody response which was as expected because it does not contain the 6 lysines on the N-terminus that are known to increase immunogenicity (Fig. 2G and H). Importantly, for all the A β derivatives the IgG antibody levels went back to baseline once immunization was discontinued as measured 22 weeks (T3) following the third immunization. This finding enhances the safety of this approach, because

should any antibody-related side effects occur, these should subside with discontinuation of therapy. A β 1-42 elicited the most robust antibody response, with the IgG levels not going back to baseline once treatment was halted (Fig. 2I and J).

Regarding IgM levels, those are known to peak 1–2 weeks following immunization and then the antibodies are cleared. Our findings confirmed this well known fact as IgM levels in all the groups decreased to preimmunization levels at the T3 bleed. Overall, these results indicate that most of our A β derivatives elicit a substantial antibody response in primates, and importantly this effect is reversible (see T3) which enhances the safety profile of this type of immunization. It should be considered optimal to have this reversibility feature designed into any vaccine that targets self-antigens and has the potential to induce autoimmune toxicity. The primates elicited a similar antibody response towards A β as we have observed in mouse models with the exception of K6A β 1-30[E₁₈E₁₉] that in BL6/SJL Tg2576 mice elicited very low IgG but high IgM response whereas in the primates both IgG and IgM levels were moderate to high.

These young primates should not have any A β deposits within the brain but we sought to determine if their antibody response altered their plasma A β 1-40 levels as an indicator of the physiological relevance of the immunization. A β 1-40 levels in plasma were measured at T0, T2 and T4 (Fig. 3; for comparison the levels of anti-A β 1-40 antibodies at the same intervals is depicted in the right panel). A β 1-40 plasma levels correlated well with both IgG (A β 1-30 [E₁₈E₁₉], $p < 0.01$; K6A β 1-30, $p = 0.02$) and IgM (A β 1-30[E₁₈E₁₉], $p < 0.001$; A β 1-42, $p < 0.03$) against A β 1-40. These findings indicate that the antibodies that are generated in these primates in response to the immunization are having a biological effect on A β in vivo.

To assess if the antibodies generated in response to the vaccine would recognize AD plaques or congophilic angiopathy, AD brain sections were stained with plasma from animals in all treatment groups. Staining of parenchymal and vascular A β deposits was observed with plasma from all the immunized groups and as expected was more prominent in bleeds with higher A β antibody levels (Fig. 4).

As one measure of general health, the animals were weighted the same day they were bled (T0–T5; Fig. 5). None of the groups lost weight during this period but two animals were euthanized during the study because of substantial weight loss: (1) One adjuvant control 6 weeks after the fourth bleed (T3) and (2) One A β 1-30[E₁₈E₁₉]-treated primate, 2 weeks after the second bleed (T1). While the cause of the weight loss is unclear there is no indication that it was related to the adjuvant or the vaccine although that possibility cannot be completely ruled out. Interestingly, significant weight increase was observed in all the immunized groups, K6A β 1-30 ($p = 0.02$), K6A β 1-30[E₁₈E₁₉] ($p = 0.01$), A β 1-30[E₁₈E₁₉] ($p = 0.03$), and A β 1-42 ($p = 0.0001$), but not in the controls ($p = 0.14$). Post hoc analysis revealed significant weight gain at T2 compared to T0 in K6A β 1-30- ($p = 0.01$), and A β 1-42-treated primates ($p = 0.0001$), and a strong trend for weight gain in K6A β 1-30[E₁₈E₁₉]-treated ($p = 0.07$) and A β 1-30 [E₁₈E₁₉]-treated primates ($p = 0.10$). The weight of those animals went back to preimmune (T0) values at subsequent intervals. Furthermore, weekly observations by a veterinarian indicated as well that the animals were in good health.

4. Discussion

Most of the immunization approaches targeting A β have been performed in transgenic models that overexpress mutated human amyloid precursor protein which results in high levels of human A β . The mice have normal endogenous levels of mouse A β as well. These models are ideal for initial screening of potential therapy but prior to clinical trials it is imperative to perform studies in animals such as primates that express normal levels of A β which has the

same sequence as human A β . One of these models is *M. murinus*, a lemur primate that develops Alzheimer's like brain pathology with age [16–18].

Our present findings in a relatively small group of young primates indicate that immunization with A β derivatives appears to be safe as a prophylactic measure. Transient weight gain was observed which indicates that the vaccines do not appear to have detrimental effects on the general health of these young animals. Importantly, anti-A β antibody levels went back to baseline when therapy was discontinued in the A β homolog treated primates but not in the A β 1-42 treated animals. These observations show that active immunization with A β derivatives is analogous to passive immunization with regard to its reversibility in plasma antibody levels when immunization is discontinued. Furthermore, it will be less likely to induce an anti-idiotypic response as the antibodies are formed endogenously. As should be expected, not all mammals react in the same way. Head et al. observed that in young beagles immunized with A β 1-42 in Titermax adjuvant, antibody titer was back to preimmune values by 12 weeks [24]. However their experimental design was different from ours with regard to dose, adjuvant and numbers of injections.

The increase of A β 1-40 antibodies in the primates often correlated with increased A β 1-40 in plasma which suggests that the antibodies generated in response to the vaccines were binding to A β in vivo. The spike in plasma levels of A β can be explained by increased half-life of bound A β in plasma and/or by antibody-mediated sequestration of A β from organs to blood.

A β T cell epitopes have not been described in lemur primates but as mid-region A β T cell epitopes have been detected in mice and humans, this type of response is more likely to occur following administration of K6A β 1-30 than with the modified A β derivatives K6A β 1-30 [E₁₈E₁₉] and A β 1-30[E₁₈E₁₉]. However, T-helper epitopes are necessary for antibody response and those often overlap with cytotoxic T cell epitopes depending on the haplotype of the individual which complicates vaccine design. With appropriate choice of adjuvant, for example alum (Th2) over QS-21 (Th1), cytotoxic T cell response can likely be contained.

Low ELISA signal in preimmune plasma or controls was occasionally detected in the antibody assays. As depicted in Figs. 2 and 3, the ELISA IgG and IgM signal at T0 or in the controls at all bleeds is either barely measurable or much lower than post-immunization. As several of the animals had a value of 0, it is more likely that the signal is specific for A β autoantibodies rather than representing a non-specific binding of the peroxidase-linked secondary antibody. We are not aware of studies that have measured A β autoantibodies in young humans but those are frequently observed in middle-aged and older individuals [25–27]. Furthermore, we have noticed an age-related increase in A β 40 autoantibodies in older human controls (age range 56–81) as well as in the A β plaque mouse model Tg2576 (unpublished results).

It is unclear why transient weight gain occurred in the immunized primates but it is interesting that they were heaviest at the T2 bleed, the time point of their highest levels of A β antibodies. This observation may suggest that A β has a role in weight regulation. Several gastrointestinal hormones and neuropeptides are known to have complex effects on appetite and energy homeostasis [28]. As A β is found in all biological fluids, it likely has both peripheral and central effects that can be altered by immunotherapy although compensatory mechanisms may only lead to transient changes.

We are not aware of other investigators using modified A β derivatives (A β 1-30[E₁₈E₁₉], K6A β 1-30[E₁₈E₁₉]) as potential AD vaccines, but studies on unaltered A β fragments as vaccine components have recently been described by others [29–32]. Also, mice have been vaccinated with a phage displaying amino acids 3–6 of the A β peptide which resulted in a modest antibody response, promoted clearance of amyloid deposits [33], and improved cognition [34], supporting our previous findings that a relatively low antibody titer is sufficient

to enhance memory and learning [5]. A different promising approach combines A β 1-15, its immunodominant B-cell epitope, with a promiscuous T-cell epitope that in wild-type mice elicits high anti-A β antibody titers without T-cell reactivity towards A β [35]. A similar approach has recently been shown to reduce amyloid burden and learning deficits in AD mouse model in the absence of A β specific cellular immune response [36]. Together, the use of A β derivatives for active immunization is likely to have a better safety profile for humans than administering full-length A β . To selectively target highly toxic forms of A β should also be beneficial although the types of antibodies generated are likely to vary between individuals. For example, the use of oligomeric A β as immunogen has been shown to reduce A β burden in transgenic mice with less microglial response than other A β peptides [37]. Potential toxicity associated with use of oligomeric A β can be avoided by using monoclonal antibodies that selectively target these A β aggregates. At least three clinical trials by Elan/Wyeth (ACC-001), Cytos/Novartis (CAD106) and Merck (V950) are ongoing on this type of active immunotherapy targeting A β . Dosing in the ACC-001 trial was recently suspended temporarily, because one patient in the Phase II trial developed skin lesions [38]. The ACC-001 vaccine is A β 1-7 linked to an immunostimulatory molecule, a mutated diphtheria toxin CRM-197, and is delivered with or without QS-21, a strong Th1-type adjuvant. It is unclear what may have caused this reaction in the patient who is currently recovering.

The main purpose of this present immunization study in young primates was twofold: (1) to assess the safety of this approach as a prophylactic measure using an animal model that is closer to humans than transgenic mice, and (2) to select one A β derivative for studies in old lemur primates. One control primate that received alum adjuvant alone and one A β 1-30[E₁₈E₁₉]-treated primate were euthanized during the study because of a substantial weight loss. It is unlikely that this condition was related to the immunization as one of the animals was a control that only received adjuvant that is approved for human use, and should therefore be considered safe. Unexplained weight loss in a few animals has occasionally been observed over the years in this colony of about 250 primates.

While we continue to monitor the young primates for any adverse reactions, we have now initiated studies in older lemurs employing K6A β 1-30 because it showed the most consistent antibody response in the young animals. As those older animals may have Alzheimer's-like neuropathology, this approach should provide invaluable information for future clinical trials on this type of A β immunotherapy that embodies the advantages of active (endogenous response) and passive (reversibility) immunization approaches.

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Fig. 1. Schedule for immunizations and bleeds. A schematic diagram depicting the timeline of the immunizations and bleeds for measurements of antibody response and A β levels.

Fig. 2.

Antibody response in lemur primates. The primates ($n = 5$ per group) were bled prior to immunization (T0). Subsequent vaccinations were at 0, 2, 6, 42, 44 and 48 weeks. Subsequent bleeds were at 3 (T1), 7 (T2), 28 (T3), 45 (T4) and 49 weeks (T5). (A, B) Very low levels of autoantibodies are observed in control animals (1:200 dilution). (C–H) All the Aβ derivatives have a moderate to high antibody response (IgG and IgM) but it is transient because 22 weeks after the 3rd injection (T3), antibody levels are similar to controls. However, antibodies are regenerated when the primates are reimmunized. (I, J) Aβ1-42 elicits an overall stronger antibody response that is maintained at moderate levels (IgG) 22 weeks (T3) after the 3rd injection. Also, more antibodies are generated when the primates are reimmunized. Treatment

and antibody type measured (IgG or IgM) is indicated in each graphs' title. The *x*-axis depicts which peptide (coated on ELISA plate) the antibodies are recognizing. The *y*-axis depicts the absorbance at 450 nm.

Fig. 3.

Plasma Aβ1-40 levels versus antibody response. A good correlation was often observed between plasma Aβ1-40 levels (left panel) and levels of anti-Aβ1-40 IgG (middle panel) or IgM (right panel) in the young lemurs. Aβ1-40 plasma levels correlated well with both IgG (Aβ1-30[E₁₈E₁₉], $p < 0.01$; K6Aβ1-30, $p = 0.02$) and IgM (Aβ1-30[E₁₈E₁₉], $p < 0.001$; Aβ1-42, $p = 0.03$) against Aβ1-40.

Fig. 4. Staining of parenchymal and vascular A β deposits with plasma from immunized primates. Representative examples of AD brain sections stained with plasma (T2; 1:100 dilution) from lemur primates immunized with (A) A β 1-30[E₁₈ E₁₉] and (B) A β 1-42. Adjacent section (C) was stained with a mixture of 6E10/4G8 monoclonal antibodies (1:1000) to reveal all A β deposits in this particular AD case. As expected, more plaques were observed in the A β 1-42 immunized primate as it had higher A β antibody levels than the animal immunized with A β 1-30[E₁₈ E₁₉] (A β 1-42 treated primate: anti-A β 1-40 = 3.3; anti-A β 1-42 = 3.3. A β 1-30[E₁₈ E₁₉]-treated primate: anti-A β 1-40 = 1.5; anti-A β 1-42 = 2.6. Values are arbitrary ELISA signals at 1:200 dilution at 450 nm). Similar titer-dependent staining was observed in the

K6A β 1-30- and K6A β 1-30[E₁₈ E₁₉]-treated groups (data not shown). Original magnification: 50 $\times$.

Fig. 5.

Weight of primates. A significant weight increase was observed in all the immunized groups, K6Aβ1-30 ($p = 0.02$), K6Aβ1-30[E₁₈ E₁₉] ($p = 0.01$), Aβ1-30[E₁₈ E₁₉] ($p = 0.03$), and Aβ1-42 ($p = 0.0001$), but not in the controls ($p = 0.14$). Post hoc analysis revealed significant weight gain at T2 compared to T0 in K6Aβ1-30- ($p = 0.01$), and Aβ1-42-treated primates ($p = 0.0001$), and a strong trend for weight gain in K6Aβ1-30[E₁₈ E₁₉] ($p = 0.07$) and Aβ1-30[E₁₈ E₁₉] ($p = 0.10$). The weight of those animals went back to preimmune (T0) values at subsequent intervals.

Table 1

Depicts the gender, age as well as the type and number of immunizations received by each of the primates.

Animal	Sex	Age (years)	Group	Number of immunizations
376	Female	1.8	Control	6
377	Male	1.8		3 ^a
402	Male	1.3		6
403	Male	1.2		6
404	Male	1.2	K6Aβ1-30	3 ^b
375	Male	1.9		6
391	Female	1.4		6
397	Female	1.4		6
399	Male	1.2		6
408	Male	1.2	K6Aβ1-30[E ₁₈ E ₁₉]	6
371	Female	2.0		6
379	Female	1.9		6
392	Female	1.4		6
401	Male	1.3		6
405	Female	1.2	Aβ1-30[E ₁₈ E ₁₉]	6
373	Female	1.9		6
398	Female	1.2		6
400	Female	1.2		6
407	Male	1.2		6
419	Male	0.7	Aβ1-42	2 ^c
372	Male	2.0		6
374	Female	1.9		6
394	Female	1.3		6
406	Male	1.2		6
412	Male	0.7		6

^aEuthanized 21 days after the 4th bleed (T3) because of substantial weight loss. The date of death was 5 months and 23 days after the third adjuvant injection.

^bDiscontinued in the study after the 4th bleed because it was required for breeding.

^cEuthanized 15 days after the 2nd bleed (T1) because of substantial weight loss. The date of death was 22 days after the 2nd immunization.