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Consequence of Aβ immunization on the vasculature of human Alzheimer's disease brain

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A major feature of Alzheimer's disease is the accumulation of amyloid-β peptide (Aβ) in the brain both in the form of plaques in the cerebral cortex and in blood vessel as cerebral amyloid angiopathy (CAA). Experimental models and human clinical trials have shown that accumulation of Aβ plaques can be reversed by immunotherapy. In this study, we hypothesized that Aβ in plaques is solubilized by antibodies generated by immunization and drains via the perivascular pathway, detectable as an increase in cerebrovascular Aβ. We have performed a follow up study of Alzheimer's disease patients immunized against Aβ42. Neuropathological examination was performed on nine patients who died between four months and five years after their first immunization. Immunostaining for Aβ40 and Aβ42 was quantified and compared with that in unimmunized Alzheimer's disease controls ($n = 11$). Overall, compared with these controls, the group of immunized patients had approximately 14 times as many blood vessels containing Aβ42 in the cerebral cortex ($P < 0.001$) and seven times more in the leptomeninges ($P = 0.013$); among the affected blood vessels in the immunized cases, most of them had full thickness and full circumference involvement of the vessel wall in the cortex ($P = 0.001$), and in the leptomeninges ($P = 0.015$). There was also a significantly higher level of cerebrovascular Aβ40 in the immunized cases than in the unimmunized cases (cortex: $P = 0.009$ and leptomeninges: $P = 0.002$). In addition, the immunized patients showed a higher density of cortical microhaemorrhages and microvascular lesions than the unimmunized controls, though none had major CAA-related intracerebral haemorrhages. The changes in cerebral vascular Aβ load did not appear to substantially influence the structural proteins of the blood vessels. Unlike most of the immunized patients, two of the longest survivors, four to five years after first immunization, had virtually complete absence of both plaques and CAA, raising the possibility that, given time, Aβ is eventually cleared from the cerebral vasculature. The findings are consistent with the hypothesis that Aβ immunization results in solubilization of plaque Aβ42 which, at least in part, exits the brain via the perivascular pathway, causing a transient increase in the severity of CAA. The extent to which these vascular alterations following Aβ immunization in Alzheimer's disease are reflected in changes in cognitive function remains to be determined.

Keywords: Alzheimer's disease; cerebral amyloid angiopathy; immunotherapy; vasculature

Abbreviations: Aβ = amyloid-β peptide; CAA = cerebral amyloid angiopathy; iAD = immunized Alzheimer's disease; MSI = Microhaemorrhage Severity Index; SMA = smooth muscle actin

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Introduction

Alzheimer's disease is the commonest cause of cognitive decline in ageing. According to the amyloid hypothesis (Hardy and Selkoe, 2002), abnormal aggregation of the amyloid-β peptide (Aβ) in the brain triggers the downstream effects of tau aggregation, microglial activation,

synaptic dysfunction and neuronal loss together ultimately resulting in cognitive decline (Albert, 1996; Graham and Lantos, 2002). Aβ accumulates in the brain in the form of extracellular aggregates in the cerebral cortex (plaques) and in the walls of blood vessels as cerebral amyloid angiopathy (CAA) (Graham and Lantos, 2002). Schenk and colleagues demonstrated that active peripheral immunization with the Aβ42 peptide in a transgenic mouse model of Aβ deposition resulted in both prevention of plaque formation and removal of existing plaques (Schenk *et al*., 1999). Experimental studies of both active and passive Aβ immunization in transgenic mice have since confirmed that removal of existing Aβ plaques can occur (Bard *et al*., 2000; Games *et al*., 2000; DeMattos *et al*., 2001; Wilcock *et al*., 2004*a*), sometimes within a matter of days (Bacskai *et al*., 2001; Wilcock *et al*., 2003) and that this is associated with cognitive benefits (Janus *et al*., 2000; Morgan *et al*., 2000; Dodart *et al*., 2002). Immunization strategies in mice can also neutralize the Aβ oligomers, which current evidence suggests are a particularly neurotoxic form of Aβ (Klyubin *et al*., 2005; Lesne *et al*., 2006).

The first clinical trial of Aβ immunization in Alzheimer's disease was initiated in 2000 by Elan Pharmaceuticals Inc. to explore tolerability and immunogenicity of active Aβ42 immunization (Bayer *et al*., 2005). In this study involving 80 patients with mild to moderate Alzheimer's disease, 64 patients received active immunization with synthetic Aβ42 (AN1792) and 16 patients received a placebo. During the 18 month study period, Aβ antibodies were generated, predominantly to the N terminus (Lee *et al*., 2005), by ~50% of the patients receiving Aβ42 (Bayer *et al*., 2005). In a subsequent larger clinical trial ($n = 372$), an inflammatory complication was identified in 6% of the patients which halted the trial (Orgogozo *et al*., 2003). Despite this setback, post-mortem studies from patients in both trials confirmed that plaque removal occurred in immunized Alzheimer's disease patients as predicted by the experimental mouse models (Nicoll *et al*., 2003, 2006; Ferrer *et al*., 2004; Masliah *et al*., 2005; Bombois *et al*., 2007).

A consistent feature of the published post-mortem cases has been the persistence of Aβ associated with the cerebral vasculature i.e. cerebral amyloid angiopathy (CAA) despite the reduction of Aβ in the form of plaques (Nicoll *et al*., 2003, 2006; Ferrer *et al*., 2004; Masliah *et al*., 2005). CAA is the accumulation of amyloid—in this context, Aβ—in the walls of arteries and arterioles in the leptomeninges and cerebral cortex (Vinters, 1987; McCarron *et al*., 1999). It occurs commonly in ageing and is usually thought to be asymptomatic. However, it can present with stroke as a result of spontaneous major intracerebral haemorrhage which is characteristically superficial (lobar) and may be multiple or recurrent. Such haemorrhages have also been observed in a mouse model of severe CAA (Winkler *et al*., 2001). Very rarely, severe CAA is associated with a rapidly progressive dementia, often accompanied by multifocal microhaemorrhages (Yamada, 2000). Aβ peptide exists in two major forms differing in length at the C terminus by two amino acids, Aβ42 and Aβ40, with Aβ42 predominating in cortical plaques and Aβ40 being the predominant form in the vasculature (Roher *et al*., 1993; Gravina *et al*., 1995). Recently, several mechanisms or pathways for Aβ elimination have been identified including: (i) enzymatic degradation by neprilysin and/or insulin-degrading enzyme (McDermott and Gibson, 1997; Iwata *et al*., 2000; Hellstrom-Lindahl *et al*., 2008); (ii) low density lipoprotein receptor-related protein 1 (LRP-1) mediated clearance of Aβ across the blood–brain barrier into the blood (Hyman *et al*., 2000; Shibata *et al*., 2000); and (iii) clearance of Aβ by drainage along perivascular pathways with interstitial fluid, blockage of which is postulated to be the cause of CAA (Weller *et al*., 1998; Preston *et al*., 2003; Nicoll *et al*., 2004; Carare-Nnadi *et al*., 2005).

In this study, we have explored the Aβ associated with the cerebral vasculature with the following hypotheses in mind: (i) Aβ immunization in Alzheimer's disease results in an increase in the severity of CAA as Aβ plaques are solubilized; (ii) this increases the amount of Aβ42 in the vessel walls, reflecting solubilisation and perivascular drainage of plaque-derived Aβ42; (iii) the increase in CAA severity is associated with an increase in the number of microhaemorrhages; and (iv) accumulation of vascular Aβ influences the structural proteins of the blood vessel wall.

Materials and Methods

Cases

We performed a clinical and neuropathological follow-up of patients who were enrolled in the initial Elan Pharmaceuticals Inc. trial of Aβ42 immunization in Alzheimer's disease (Holmes *et al*., 2008). As part of the study, patients and their carers were invited to consent to post-mortem neuropathology. The study received ethical approval from Southampton and South West Hampshire Local Research Ethics Committees (Reference No: LRC 075/03/w). We obtained post-mortem brains for neuropathology from nine patients, all of whom had received Aβ42 plus adjuvant and who died between 4 and 64 months after the first immunization dose (Table 1). Assessment of pathology associated with dementia was performed by examining histological sections of frontal, temporal, parietal and occipital lobes, corpus striatum, thalamus, midbrain, pons, medulla and cerebellum. Paraffin sections were stained with haematoxylin and eosin (H&E), Luxol fast blue/cresyl violet and modified Bielschowsky silver impregnation. Selected sections were immunostained for Aβ, tau and α-synuclein. In one case, the neuropathological assessment indicated a diagnosis of progressive supranuclear palsy, on the basis of neuronal tangles predominantly in the brainstem and basal ganglia, sparse in the cerebral neocortex and absent from the hippocampus; the neuropathological diagnosis was supported on review of the clinical records. In the remaining eight cases, designated immunized Alzheimer's disease, the distribution of tau pathology was completely different, and typical of Alzheimer's disease, with predominantly cortical tangles and severe hippocampal involvement. These cases therefore showed appearances consistent with Alzheimer's disease (Braak stage V/VI) in which Aβ components

of the pathology had been influenced by Aβ immunization as previously described (Nicoll *et al.*, 2003, 2006) (Table 1).

Controls

Paraffin sections from archival cases of Alzheimer's disease from the neuropathology service in Southampton General Hospital were used as unimmunized Alzheimer's disease controls ($n = 11$). All patients had a history of progressive dementia and satisfied Consensus Criteria for Alzheimer's disease (The National Institute on Aging, 1997) (Table 2). In addition, a case with severe CAA-associated dementia from the archives of Southampton General Hospital was included as a positive control for assessment of the CAA-associated microhaemorrhages.

Immunohistochemistry

Primary antibodies

For the purposes of this study, sections of superior and middle temporal gyrus, middle frontal gyrus and cingulate gyrus were immunostained with antibodies specific for Aβ40 (clone 2G3) and Aβ42 (clone 21F12) provided by Elan Pharmaceuticals Inc. (USA) (Johnson-Wood *et al.*, 1997). Blood vessel wall components were assessed by immunostaining for endothelial cells (CD31, clone 1A10, Novocastra, UK), collagen IV (clone Col-94, Sigma, UK), laminin (rabbit anti-laminin, Sigma, UK) and smooth muscle actin (SMA; clone 1A4, DakoCytomation, UK). Microvascular lesions were characterized by immunohistochemistry for astrocytes (GFAP, clone 6F2) and microglia/macrophages (CD68, clone PG-M1), both antibodies from Dako (Glostrup, Germany).

Immunohistochemistry

The Alzheimer's disease and immunized Alzheimer's disease cases were stained together in batches for each antibody. Immunohistochemistry was performed using the appropriate antigen retrieval methods for each primary antibody. Biotinylated secondary antibodies, normal serum and avidin-biotin complex were from Vector Laboratories (Peterborough, UK). Immunohistochemistry was carried out by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC, UK) with 3,3′ diaminobenzidine (DAB) as chromogen and 0.05% hydrogen peroxide as substrate. All the sections were dehydrated before mounting in DePeX (BDH Laboratory Supplies, UK). Sections incubated in the absence of the primary antibody were included as negative controls.

Quantification

All quantification was performed blinded to the experimental group and identity of the cases and was performed on sections of superior and middle temporal gyrus, middle frontal gyrus and cingulate gyrus.

Table 1 Characteristics of immunized Alzheimer's disease cases and Aβ42 parenchymal load

Case	Gender	Age	Parenchymal Aβ42 load (%)[a]	Braak stage[b]	Dementia duration (years)	AN1792 dose (μg)[b]	Number of injections	Mean antibody response (ELISA units)[b]	Survival time from first immunization dose (months)[b]
1	F	74	1.26	VI	6	50	5	1:119	20
2	M	83	3.12	V	11	50	3	<1:100	4
3	M	63	5.67	VI	6	225	4	<1:100	41
4	F	71	4.68	VI	10	225	8	1:4072	44
5[c]	M	79	0.75	n/a (PSP)	6	50	8	<1:100	51
6	M	81	3.32	VI	7	50	8	1:1707	57
7	M	82	0.05	VI	6	50	8	1:4374	60
8	M	63	0.36	VI	10	50	8	1:6470	64
9	M	81	2.71	VI	11	225	7	1:491	63

n/a = non-applicable.
[a]Aβ42 load in sections of superior and middle temporal gyrus, middle frontal gyrus and cingulate gyrus. [b]Holmes *et al.*, Lancet 2008; 3172: 216–23. [c]Case not included in the statistical analysis.

Table 2 Characteristics of Alzheimer's disease controls and Aβ42 parenchymal load

Case	Gender	Age	Parenchymal Aβ42 load (%)[a]	Braak stage	Dementia duration (years)
1	F	80	4.58	V	3
2	F	88	4.31	VI	Several years[b]
3	F	72	4.32	VI	7
4	F	78	6.84	VI	Several years[b]
5	M	73	4.53	VI	Several years[b]
6	F	72	5.76	VI	1
7	F	85	5.25	VI	16
8	F	84	2.50	VI	5
9	F	65	5.71	VI	Several years[b]
10	F	88	4.12	VI	5
11	M	79	5.86	V	8

[a]Aβ42 load in sections of superior and middle temporal gyrus, middle frontal gyrus and cingulate gyrus. [b]Precise duration not known.

Cerebral Amyloid Angiopathy

CAA severity was quantified in sections of the immunized and unimmunized Alzheimer's disease cases in the different regions of cerebral cortex described above. The sections were immunostained separately with either Aβ42 or Aβ40 antibodies. The immunostained cortical vessels were counted (×10 microscope objective) using a graticule; the data are presented as a number of stained cortical vessels per 10 fields of cortex. The stained leptomeningeal blood vessels were counted (×10 microscope objective) throughout the section and the data presented as a percentage of the total number of meningeal vessels in the section.

In addition, 2 degrees of severity of involvement of each blood vessel wall by Aβ accumulation were noted: (i) partial staining; or (ii) full staining (i.e. staining of both the full thickness and the full circumference of the blood vessel wall).

Aβ42 plaque load

Plaque load (%) was quantified in the cerebral cortex in the same Aβ42 immunostained sections as those in which the CAA was quantified. The percentage area of cortex stained for Aβ42 (excluding the vascular staining) was measured using Image J 1.37v software (developed by Wayne Rasband, NIH, USA).

Perl's Prussian blue staining

The sections of frontal, temporal and cingulated gyrus were dewaxed, rehydrated and treated for 10 min with a freshly prepared Perl's reagent: 2% potassium ferrocyanide and 2% hydrochloric acid prepared in distilled water. Then the slides were counterstained with 0.1% neutral red, dehydrated and mounted in DePeX.

Microhaemorrhages and microvascular lesions

Microhaemorrhages were assessed using sections stained with Perl's Prussian Blue stain for iron. The haemorrhages were counted using a scoring system similar to that described in a mouse model (Pfeifer *et al*., 2002). A score of 1 was given to a cluster consisting of 1–3 detectable particles of haemosiderin, a score of 2 to a cluster of 4–10 particles and a score of 3 to a cluster of 10 or more detectable particles. The Microhaemorrhage Severity Index (MSI) for each section was calculated as: number of clusters × cluster score. For each case the mean MSI is expressed per 50 fields of cortex (×10 objective).

Microvascular lesions, which could represent either old microinfarcts or old microhaemorrhages, were defined as microscopic foci of cortical destruction associated with a microglial and astrocytic reaction and were quantified in large whole hemisphere H&E stained sections taken at the level of the mamillary bodies and therefore including temporal, parietal and frontal lobes. The data are expressed as the number of microvascular lesions per 50 fields of cortex (×10 objective).

Collagen IV, laminin and SMA

Computerized image analysis was performed on sections immunostained with antibodies specific for collagen IV, laminin and SMA. For each antibody the number of stained vessels was counted at ×10 objective magnification in 15 consecutive fields and separately in the underlying white matter, using Image J 1.37v software. An average score for the protein load (pixels) per stained vessel was obtained by dividing in each field the stained area (in pixels) by the number of stained vessels.

CD31

The CD31 staining was analysed by use of Image J 1.37v on images taken at objective magnification ×20. Thirty vessels were assessed per brain section and data are expressed as the percentage of CD31 per stained vessel.

Statistical analysis

The distributions of Aβ42 and Aβ40 stained vessels were summarized using geometric and arithmetic means and ranges, and were found to be close to normally distributed after taking logs: they were compared between unimmunized Alzheimer's disease and immunized Alzheimer's disease groups on the log scale uncontrolled and controlled for age and gender in an analysis of covariance. Estimated differences between means of the stained vessels on the log scale were back transformed to give ratios of geometric means and their 95% confidence intervals (CI). P-plots of the standardized residuals (not shown) from the analyses of covariance on the log scale confirmed approximate normality. The CAA severity as partial or full staining of the blood vessel wall for Aβ42 and Aβ40 often resulted in zero values so that logs could not be taken: levels were compared between unimmunized Alzheimer's disease and immunized Alzheimer's disease groups using exact Mann–Whitney U-test. Spearman's correlation between Aβ42 and Aβ40 levels were estimated and exact tests carried out specific to the cortex and leptomeninges and each group. The parenchymal Aβ42 and the microhaemorrhages and microvascular lesions were compared between the two groups using exact Mann–Whitney U-test. The assessment of the vascular proteins in grey and white matter were approximately normally distributed and were compared between groups using two sample *t*-tests. All statistical tests were carried out in SPSS 14.0.

Results

Quantification of cerebral amyloid angiopathy

Case 5, which had a post-mortem neuropathological diagnosis of PSP rather than Alzheimer's disease, had very little Aβ in the cerebral vasculature, as would be expected because CAA is not a feature of that disease. This case has therefore been omitted from the subsequent analysis.

Overall, compared with the unimmunized Alzheimer's disease controls, the immunized Alzheimer's disease group had approximately 14 times as many blood vessels containing Aβ42 in the cerebral cortex ($P<0.001$) and approximately seven times more in the leptomeninges ($P=0.013$) (Fig. 1A and B, Table 3). In addition, significantly more blood vessels contained Aβ40 in the immunized cases than in the unimmunized controls (cortex: $P=0.009$; leptomeninges: $P=0.002$) (Fig. 1A and B, Table 3). The increased levels of Aβ42 and Aβ40 were higher after controlling for age and gender and with greater statistical significance (Table 3), suggesting that these differences were not due to these demographic factors. There was a correlation between Aβ40 and Aβ42 in both the cortical and meningeal vasculature in the immunized cases (cortex: $r=0.929$, $P=0.002$;

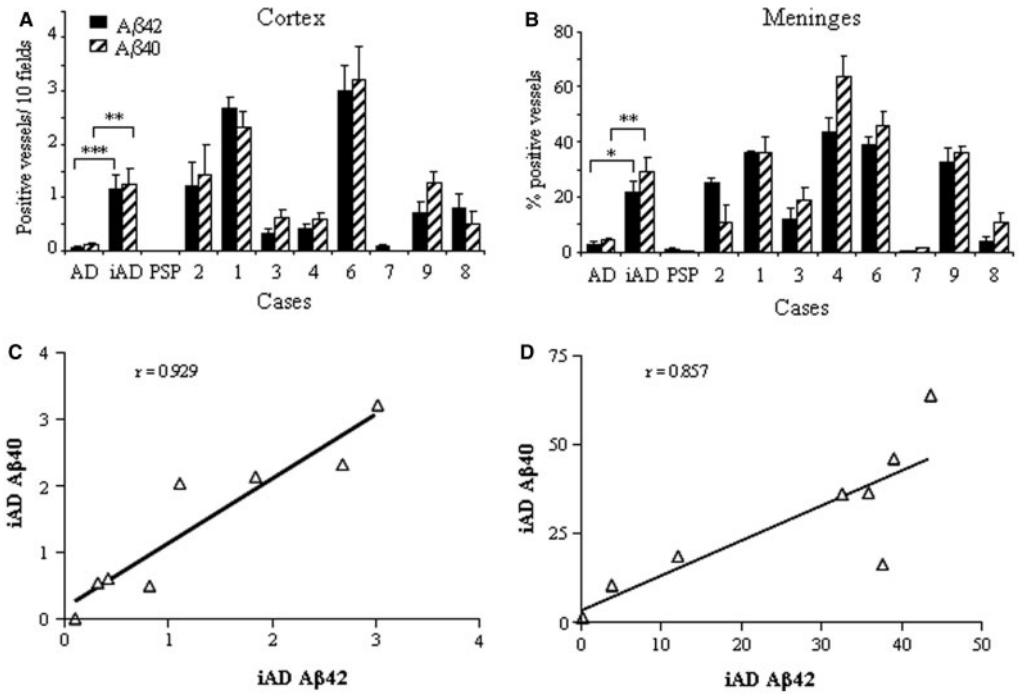

Fig. 1 Quantification of Aβ42 and Aβ40 vascular load in the cerebrum of immunized Alzheimer's disease (iAD) cases ($n = 8$) and unimmunized Alzheimer's disease (AD) controls ($n = 11$). For each graph, the mean values ± SEM for the Alzheimer's disease and immunized Alzheimer's disease groups are shown. In addition, the values for the individual immunized Alzheimer's disease cases are plotted from left to right in order of survival time since immunization (from 4 to 64 months) to show the marked variation. (**A**) In the cortex, the immunized Alzheimer's disease cases have a significantly higher density of vessels immunostained for Aβ42 ($P < 0.001$) and Aβ40 ($P = 0.009$) compared with Alzheimer's disease controls. (**B**) In the leptomeninges, the immunized Alzheimer's disease cases have a significantly higher proportion of vessels immunostained for both Aβ42 ($P = 0.013$) and Aβ40 ($P = 0.002$) compared with Alzheimer's disease controls. In the immunized Alzheimer's disease cases, there is a significant correlation between Aβ42 and Aβ40 in the cortex ($P = 0.002$) (**C**) and the leptomeninges ($P = 0.011$) (**D**). One of the immunized patients in whom the neuropathological diagnosis was PSP rather than Alzheimer's disease and was excluded from the assessment of correlation.

Table 3 Comparison of vascular Aβ42 and Aβ40 staining in the cortex and leptomeninges by analysis of covariance uncontrolled and controlled for age and gender

		Alzheimer's disease ($n = 8$)	immunized Alzheimer's disease ($n = 11$)	Uncontrolled		Controlled for age and gender	
				$\frac{iAD}{AD}$ geometric means (CI)	*P*	$\frac{iAD}{AD}$ geometric means (CI)	*P*
Cortex	Aβ42	0.056 (0.071) 0.02–0.21	0.808 (1.289) 0.10–3.02	14.44 (5.80–35.92)	<0.001	14.70 (4.67–46.29)	<0.001
	Aβ40	0.071 (0.134) 0.01–0.42	0.690 (1.416) 0.01–43.58	9.65 (1.92–48.61)	0.009	13.48 (1.70–107.13)	0.017
Leptomeninges	Aβ42	1.872 (3.385) 0.28–10.42	13.182 (25.603) 0.26-43.58	7.04 (1.61–30.77)	0.013	12.10 (1.81–80.96)	0.014
	Aβ40	2.786 (4.413) 0.23–11.30	19.042 (28.583) 1.43–63.69	6.84 (2.18–21.41)	0.002	13.76 (3.57–52.87)	0.001

Figures are geometric mean (arithmetic mean), min to max. AD = Alzheimer's disease; iAD = immunized Alzheimer's disease.

leptomeninges: $r = 0.857$, $P = 0.011$) which was less strong in unimmunized cases (cortex: $r = 0.568$, $P = 0.072$; leptomeninges: $r = 0.656$, $P = 0.032$) (Fig. 1C and D).

In addition to there being more blood vessels stained for Aβ in the immunized Alzheimer's disease cases, individual blood vessels were more severely affected (Table 4). Most of the blood vessels in the immunized cases had staining for Aβ40 and Aβ42 involving both the full thickness and full circumference of the vessel wall (Fig. 2), a finding which was relatively infrequent in the unimmunized Alzheimer's disease controls. Compared with the controls, the immunized cases had substantially greater numbers of blood vessels with Aβ42 staining of full thickness and full circumference of the vessel wall in both the cortex ($P = 0.001$) and in the leptomeninges ($P = 0.015$).

Comparison of CAA and Aβ42 plaque load

Although the number of cases available for study was small for this type of analysis and there was variation both between cases and between the different anatomical regions

Table 4 Analysis of the CAA severity assessed by immunostaining for Aβ42 and Aβ40 in the immunized Alzheimer's disease cases and Alzheimer's disease controls

		Cortex			Leptomeninges		
		Alzheimer's disease ($n = 11$)	immunized Alzheimer's disease ($n = 8$)	*P*	Alzheimer's disease ($n = 11$)	immunized Alzheimer's disease ($n = 8$)	*P*
Aβ42	Partial	0.03 (0.04) 0.00–0.08	0.10 (0.10) 0.10–0.24	0.048	0.34 (1.34) 0.00–3.58	4.29 (4.99) 0.13–11.51	0.062
	Full	0.02 (0.03) 0.00–0.12	0.83 (1.19) 0.02–2.78	0.001	0.97 (2.05) 0.00–8.47	24.95 (20.62) 0.13–41.69	0.015
Aβ40	Partial	0.01 (0.02) 0.00–0.08	0.06 (0.06) 0.00–0.13	0.071	2.10 (1.45) 0.00–2.83	2.65 (4.09) 0.37–10.53	0.088
	Full	0.10 (0.11) 0.00–0.33	1.27 (1.35) 0.00–3.11	0.004	1.43 (2.96) 0.00–11.30	21.83 (24.49) 0.00–61.22	0.021

Figures are median (mean), min-max. 'Partial' = partial staining of the blood vessel wall for Aβ; 'Full' = staining of both the full thickness and full circumference of the blood vessel wall.

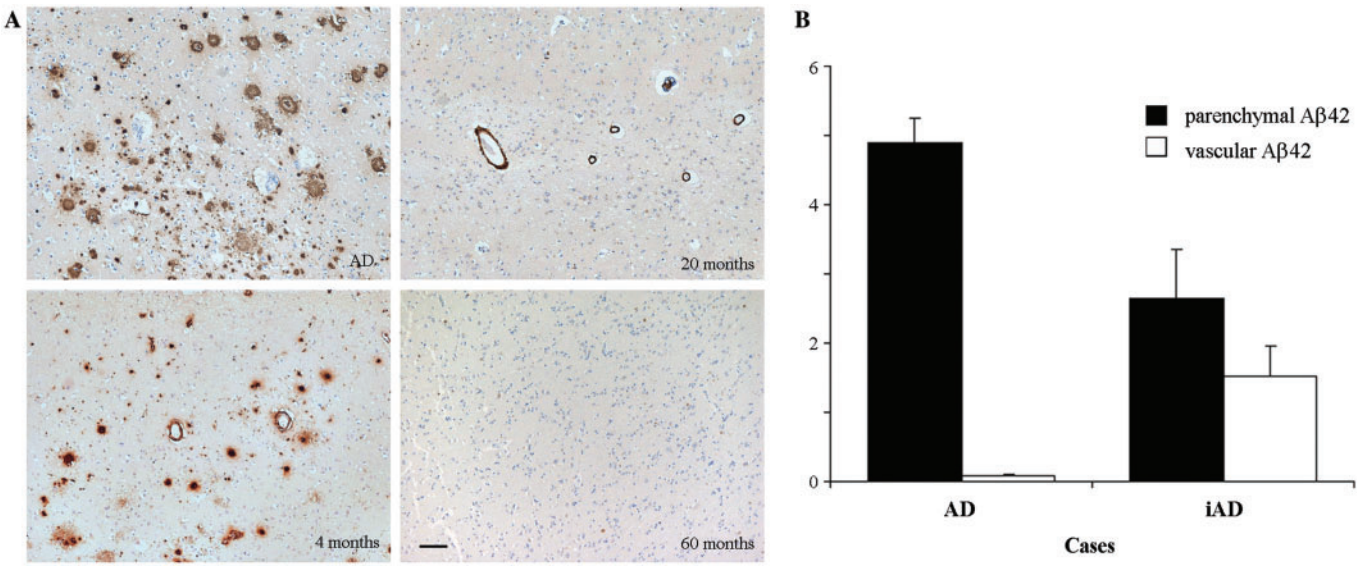

Fig. 2 (**A**) Illustration of the relative distribution of Aβ42 in the parenchyma versus the vasculature at different times after Aβ42 immunization. In the unimmunized Alzheimer's disease cases, almost all the Aβ42 is in the form of plaques with very little in the vasculature. Four months after immunization, there is relatively abundant vascular Aβ42 in the presence of 'moth-eaten' plaques. At 20 months post-immunization, vascular Aβ42 involving the full thickness and full circumference of the blood vessel walls is observed with no parenchymal Aβ42. At 60 months post-immunization, there is little Aβ42 either in plaques or vessels. Scale bar = 50 μm. (**B**) Quantification of parenchymal Aβ42 and vascular Aβ42 in Alzheimer's disease and immunized Alzheimer's disease cases. The immunized Alzheimer's disease group shows a significantly lower load of parenchymal Aβ42 ($P = 0.020$) and a significantly higher level of vascular Aβ42 ($P < 0.001$).

of cerebral cortex within individual immunized cases, subjective assessment of the histological appearance suggested a time-course of events following immunization (Fig. 2). Prior to immunization (i) Aβ42 is located predominantly in plaques with little Aβ42 in the vasculature; then following immunization (ii) plaques have a moth-eaten appearance and abundant Aβ42 is detectable in the vessel walls; (iii) at a later stage, Aβ42 plaques have been removed from the cortex but a substantial quantity of Aβ42 remains in the walls of the vasculature; and in the final stage (iv) Aβ42 has been removed from both plaques and blood vessel walls. This putative sequence of events is illustrated in sequence in Fig. 2. The comparison between the parenchymal and the vascular Aβ42 shows a significant lower of parenchymal Aβ42 ($P = 0.020$) after immunization associated with a significant higher level of vascular Aβ42 ($P < 0.001$) (Fig. 2B).

Microhaemorrhages and microvascular lesions

Haemosiderin clusters resulting from parenchymal microhaemorrhages were quantified in sections stained with Perl's stain for iron (Fig. 3A–C). The immunized Alzheimer's disease cases were compared with unimmunized Alzheimer's disease controls and an unimmunized patient who developed a rapidly progressive dementia associated with severe CAA, in order to put the findings into the context of very

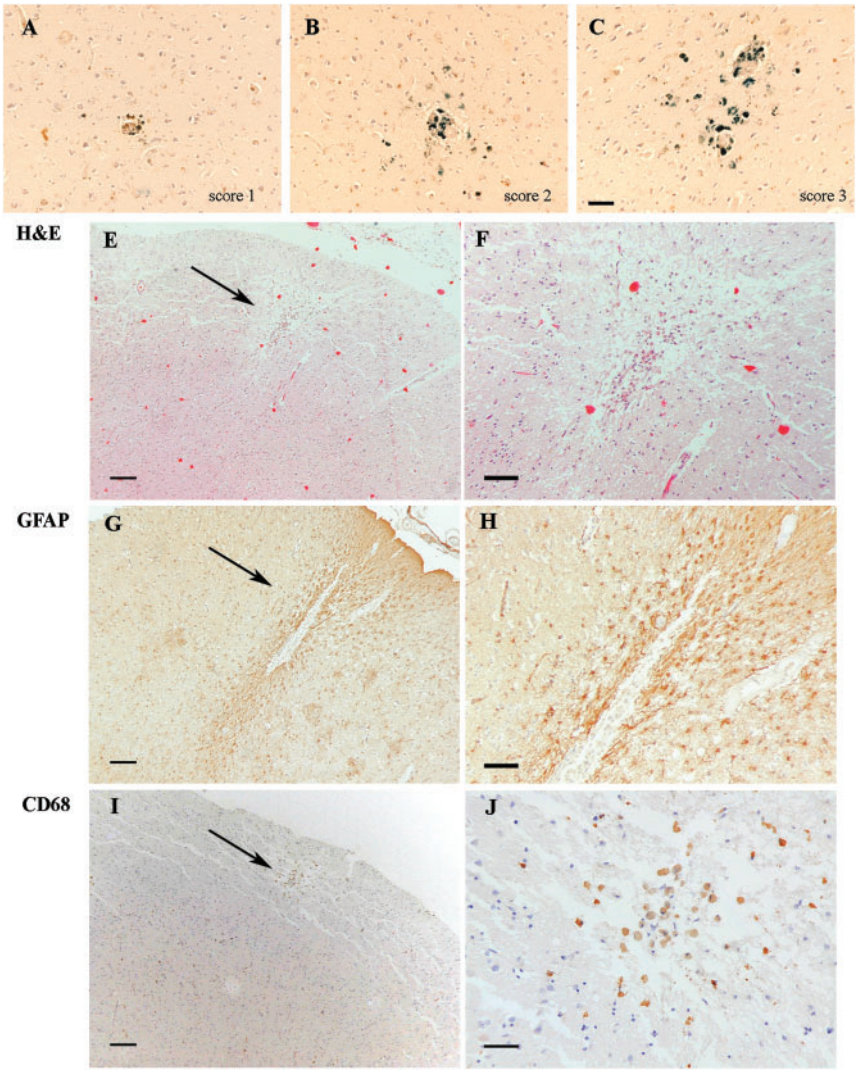

Fig. 3 Illustration of microhaemorrhages and microvascular lesions in the immunized Alzheimer's disease cases. Microhaemorrhages were assessed in sections stained with Perl's stain for iron and classified as (**A**) score I, (**B**) score 2 or (**C**) score 3, used to derive the Microhaemorrhage Severity Index. IIIlustration of a microvascular lesion, which could represent an old microhaemorrhage or an old micro-infarct, in immunized Alzheimer's disease case I: (**E** and **F**) on H&E staining. Characterization of a microvascular lesion (**G** and **H**) by the presence of abundant surrounding activated astrocytes (immunohistochemistry for GFAP) and (**I** and **J**) activated macrophages within the lesion (immunohistochemistry for CD68). H&E = haematoxylin and eosin staining. Scale bar: (**A–C** and **J**) = 50 μm; (**F** and **H**) = 100 μm; (**E**, **G** and **I**) = 200 μm.

severe symptomatic CAA. Very little Perl's positive staining was identified in the unimmunized Alzheimer's disease cases (MSI = 0.04). The mean MSI score for the immunized cases was 1.01 ($P = 0.021$); however, there was considerable inter-individual variation (median = 0.25). Case 9 had the highest MSI, more than 100 times the severity of that in unimmunized cases, but even excluding this case as a potential outlier the relationship remained significant ($P = 0.043$). To put the microhaemorrhage severity into context even case 9 had less than one-third of the MSI value in the CAA-associated dementia case (6 versus 20 MSI per 50 fields.).

Microvascular lesions, foci of cortical parenchymal damage which could have been due either to old micro-infarcts or old microhaemorrhages from which the iron pigment has been resorbed, were very infrequent in the unimmunized Alzheimer's disease controls. Five of the eight immunized cases had microvascular lesions (Fig. 3E–J) ranging from 0.05 to 0.54 lesions per 50 fields of cortex (Fig. 4B); the median value of the immunized cases differed significantly from that of the unimmunized controls (0.06 versus 0.00; $P = 0.033$).

Overall, the immunized Alzheimer's disease cases with the microhaemorrhages and microvascular lesions did not differ from those without microhaemorrhages and microvascular lesions in terms of either the CAA severity or evidence of plaque clearance.

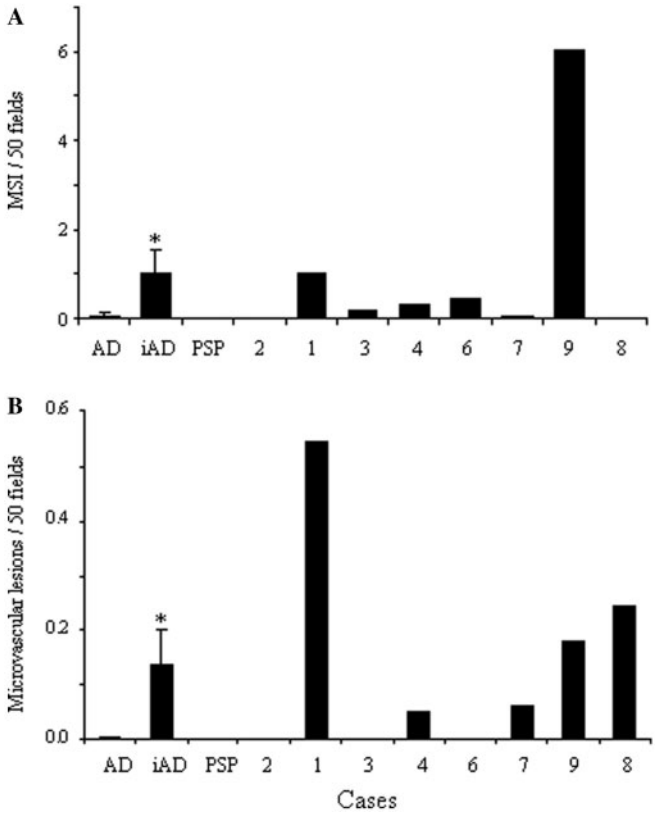

Fig. 4 Quantification of microhaemorrhages and microvascular lesions in the immunized Alzheimer's disease cases and Alzheimer's disease controls. For each graph, the mean values ± SEM for the Alzheimer's disease and immunized Alzheimer's disease groups are shown. In addition, the values for the individual immunized cases are plotted to show the marked variation. (**A**) Quantification of microhaemorrhages assessed by Perl's staining in the cerebral cortex shows significantly more microhaemorrhages in immunized Alzheimer's disease versus Alzheimer's disease ($P = 0.021$), although there is considerable variation among the immunized cases. (**B**) Quantification of microvascular lesions assessed on H&E stained sections in the cerebral cortex shows a significant difference (immunized Alzheimer's disease versus Alzheimer's disease, $P = 0.033$), although there is also considerable variation among the immunized Alzheimer's disease cases. The immunized Alzheimer's disease cases are arranged from left to right in order of survival time following first immunization dose (from 4 to 64 months). In one of the immunized patients the neuropathological diagnosis was PSP rather than Alzheimer's disease. MSI = microhaemorrhage severity index.

Effects on blood vessel wall structure

Four different proteins of the blood vessel wall (CD31, SMA, laminin and collagen IV) were studied to investigate whether the increase of Aβ within the cerebral vasculature following immunization influenced blood vessel wall structure. Although subjective histological analysis suggested higher vascular loads of laminin and SMA in some immunized Alzheimer's disease cases (Fig. 5), significant differences between the unimmunized and immunized groups were not achieved on quantification of the staining (Table 5).

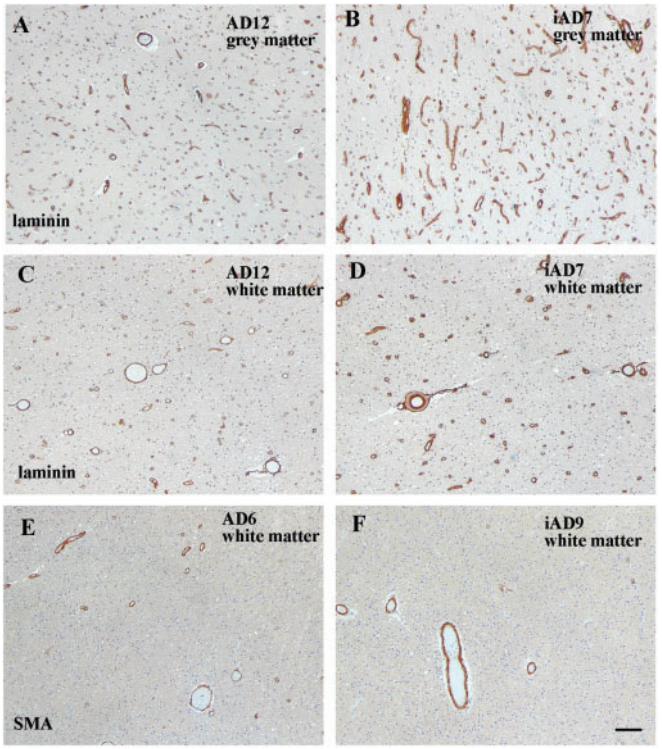

Fig. 5 Illustration of immunostaining in the immunized Alzheimer's disease and Alzheimer's disease cases for vascular laminin in the grey matter (**A** and **B**) and in the white matter (**C** and **D**) showing more in immunized Alzheimer's disease. Illustration of SMA in the vasculature of the white matter of the immunized Alzheimer's disease versus Alzheimer's disease (**E** and **F**) showing more in immunized Alzheimer's disease. Scale bar = 100 μm.

Discussion

Following active immunization of Alzheimer's disease patients with Aβ42, there is considerable variability in the response in terms of the pathological changes. The extent of Aβ plaque clearance is patchy and variable, correlating to some extent with the time since the first immunization dose and the mean Aβ antibody response in the 18 months after immunization (Holmes *et al*., 2008). Here, despite the reduction in plaque load, we provide evidence of an increase in the quantity of Aβ associated with the vasculature in the form of CAA. CAA was more severe in both cerebrocortical and overlying leptomeningeal blood vessels in the immunized Alzheimer's disease cases. This divergence, with a lower plaque burden and an increase in CAA severity, has been clearly demonstrated in immunized APP transgenic mice in which elegant time course studies have shown that as plaques are removed the CAA severity increases (Wilcock *et al*., 2004*a*; Prada *et al*., 2007). The opportunity to observe such a time course is of necessity limited in our human post-mortem series, but as the

Table 5 Assessment of the vascular proteins in the white and grey matter of the immunized Alzheimer's disease and Alzheimer's disease cases

Protein		Alzheimer's disease ($n = 11$)	Immunized Alzheimer's disease ($n = 8$)	iAD−AD difference (95%CI)	*P*
%CD31/vessel	Grey	28.4 (6.1)	27.9 (3.6)	−0.5 (−5.6–4.6)	0.847
	White	31.5 (6.0)	30.2 (5.9)	−1.4 (−7.2–4.5)	0.631
SMA (px)/vessel	Grey	850.7 (176.4)	944.9 (134.7)	94.1 (−63.2–251.5)	0.224
	White	888.4 (242.0)	1117.1 (284.6)	228.8 (−26.5–484.0)	0.076
Laminin (px)/vessel	Grey	246.4 (28.1)	271.8 (33.9)	25.4 (−4.62–55.4)	0.092
	White	362.3 (94.5)	436.4 (102.0)	74.1 (−21.6–169.8)	0.121
Collagen IV (px)/vessel	Grey	135.3 (19.0)	153.5 (30.2)	18.2 (−5.5–42.0)	0.124
	White	259.4 (43.7)	290.8 (98.5)	31.4 (−52.8–115.6)	0.421

Figures are mean (SD).

sequence of images in Fig. 2A shows, it certainly appears that this biological phenomenon is also relevant to immunized human Alzheimer's disease. The rise in CAA severity accompanying the fall in plaque load is also consistent with the hypothesis that plaque Aβ is solubilized by binding of the Aβ antibody generated by the immunization, allowing Aβ to diffuse to the vasculature and with the concept that one of the routes of elimination of Aβ from the brain involves diffusion along perivascular basement membranes (Weller *et al.*, 1998, 2008; Weller, 2005; Carare *et al.*, 2008). Indeed, although not assessed in our study, unusually high levels of soluble Aβ have been detected in the brains of two other Alzheimer's disease patients immunized with Aβ42 (Patton *et al.*, 2006). However, our data do not exclude possibility that plaque-associated Aβ is degraded rather than 'cleared', and that increases in CAA occur independently of what is occurring with plaque-associated Aβ. It is known that some plaque clearance is due to phagocytosis by microglia (Schenk *et al.*, 1999; Nicoll *et al.*, 2003, 2006; Wilcock *et al.*, 2004*b*) which is not likely to be relevant to changes in CAA. Other possible mechanisms by which Aβ derived from plaques could be transported to the vasculature include: binding to apoE (Nicoll *et al.*, 2006), binding to immunoglobulins to form immune-complexes (Bard *et al.*, 2000) or as soluble Aβ diffusing through the neuropil (Patton *et al.*, 2006).

There is evidence that apolipoprotein E (*APOE*) genotype influences CAA in Alzheimer's disease with more severe CAA in APOE ε4 carriers (Greenberg *et al.*, 1995; Kalaria *et al.*, 1996; Premkumar *et al.*, 1996; Chalmers *et al.*, 2003). However, the relationship between plaque load and CAA severity in individuals with different *APOE* genotypes is still unclear (Love *et al.*, 2003). Some of the differences observed between the unimmunized and immunized Alzheimer's disease cases in our study might therefore have been due to different *APOE* genotypes. Due to ethical and legal constraints, the *APOE* genotypes could not be determined. This is a limitation of the study; however, even if the *APOE* genotypes had been available, the number of cases in this study would be too small to reach definitive conclusions as to the influence of genotype. In addition, the higher level of CAA severity in the immunized Alzheimer's disease group compared with the control group is way beyond anything accountable for by any possible differences between the groups in *APOE* genotypes. The difference in the pattern of Aβ distribution is reinforced by the relatively low plaque loads in the immunized group, despite the higher CAA severity (Fig. 2). Furthermore, cortical as well as leptomeningeal vessels show the same effect after immunization whereas *APOE* genotype has its major effect on capillaries (Olichney *et al.*, 2000; Thal *et al.*, 2002).

We specifically studied the effects of immunization on the distribution of Aβ42 in relation to the vasculature, as in Alzheimer's disease Aβ42 is predominantly located in plaques with very little in the vasculature (Roher *et al.*, 1993; Gravina *et al.*, 1995). Examination of the immunized cases provided us with the opportunity to test the hypothesis that solubilization of plaque Aβ42 following Aβ42 immunization might specifically increase the amount of Aβ42 associated with the vasculature. Indeed, we did find substantially more Aβ42 associated with the vasculature in the immunized patients. In some of the immunized cases, this gave rise to the unusual images of full thickness, full circumference staining of vessel walls for Aβ42 in the absence of Aβ42 plaques in the surrounding cortical parenchyma (Fig. 2). This extensive labelling of blood vessels for Aβ42 in the absence of plaques was not detected in the unimmunized subjects and appears to be a striking example of the changes prompted by the immunotherapy. However, there was also an equally substantial increase in the density of vessels containing Aβ40 with a strong correlation of Aβ40 with Aβ42, consistent with the notion that in the cerebral vasculature Aβ42 acts as a seed to promote aggregation of Aβ40 (Alonzo *et al.*, 1998). This finding supports the idea that active immunization with Aβ42 peptide also solubilizes Aβ40 from the parenchyma (Nicoll *et al.*, 2006; Patton *et al.*, 2006) as the antibodies generated are mainly directed

against the N-terminal part of the Aβ peptide which is shared by Aβ40 and Aβ42 (Lee *et al.*, 2005).

There are two key variables which may influence the response to immunization, firstly the time since first immunization dose, which ranged in this series from 4 months to 5 years, and secondly the immune response of the patient to the active immunization with Aβ42 (Holmes *et al.*, 2008). In the immunized Alzheimer's disease cases with a long survival time (5 years) and high immune response (cases 7 and 8), both CAA and plaques were virtually absent from the brain, raising the possibilities that (i) the increase in CAA may be a transient phenomenon; (ii) the kinetics of Aβ clearance from the parenchyma and from the vasculature may be different, as observed with *in vivo* imaging in mouse experiments (Prada *et al.*, 2007); and (iii) complete clearance of Aβ from the parenchyma and the vasculature can occur (Prada *et al.*, 2007) in association with a strong immune response over a prolonged time. Direct evidence for eventual clearance of Aβ from the vasculature following immunization is not available from animal experiments, possibly due to the timescale involved (5 years time post-immunization is longer than the lifespan of a transgenic mouse). Therefore this suggestion, based as it is on a small number of immunized Alzheimer's disease cases, remains somewhat speculative.

As well as an increase in CAA severity following immunization, we have identified evidence for an increase in the density of cortical microhaemorrhages and microvascular lesions. Consistent with our findings, an increase in microhaemorrhages in a transgenic APP mouse model with particularly severe CAA was observed following immunization (Pfeifer *et al.*, 2002). However, to put this into context, it is important to note that the cortical volume affected by microhaemorrhages, even in the most severely affected case, is very low and considerably lower than the natural disease of rapidly progressive dementia with CAA-associated microhaemorrhages (11.24 clusters per 50 fields representing 0.35% of the parenchyma). Due to uncertainty about the pathogenesis of the microhaemorrhages and the evolution of their histological appearance over a period of several years, we also assessed the vascular pathology by quantifying microvascular lesions, which may represent either old microhaemorrhages or old microinfarcts. Microvascular lesions also occurred with a higher density in the immunized Alzheimer's disease group than the Alzheimer's disease controls.

Nevertheless, there was no correlation between the severity of CAA and the number of microhaemorrhages or microvascular lesions (data not shown). This is not altogether surprising because we were limited in the timepoints sampled and these features are likely to have different dynamics. The CAA severity is that present at the time the patient died, whereas the microhaemorrhages/microvascular lesions are likely to be the result of the history of vascular damage over the preceding months or years. Our hypothesis about the dynamics of the process, based on animal models, suggests that at an earlier stage, while the plaques were being cleared from the brain that patient may have developed a high level of CAA, and consequently acquired the microvascular lesions at that time. However, this is speculation and current limitations in the assessment of these processes preclude direct testing of this hypothesis in the human brain.

Severe CAA can present with a stroke due to a large lobar intracerebral haemorrhage but no such pathology was identified in the cases we have examined. Orgogozo *et al.* (2003) examined the imaging scans of patients in the later trial of active immunization with Aβ42. There were two patients in the immunized group and one in the placebo group who developed a cerebral haemorrhage. One haemorrhage in the immunized group was a deep intracerebral haemorrhage and the other was lobar, of typical CAA-type (Orgogozo *et al.*, 2003). Therefore it seems that major CAA-related haemorrhages are not a common feature of the response of the Alzheimer's disease brain to Aβ42 immunization.

In Alzheimer's disease, a variety of morphological alterations in the vasculature have been reported including degeneration of the smooth muscle cells, atrophy of the endothelial cells and a thickening and local disruption of the basement membrane (Farkas and Luiten, 2001). In addition, CAA is also characterized by loss of smooth muscle cells in the vicinity of Aβ deposits as well as changes in the extracellular matrix proteins of the basement membrane (Zhang *et al.*, 1998). The increased severity of CAA following immunization, even if only transient, may accelerate the damage to the vasculature induced by the disease. We investigated these possible consequences by immunohistochemistry to assess endothelial cells and smooth muscle cells as well as the main extracellular matrix proteins of blood vessels.

The immunization did not appear to modify the endothelial cells or collagen IV protein, despite both being affected in Alzheimer's disease (Kalaria and Hedera, 1995; Tian *et al.*, 2006). However, following Aβ immunization, there was a trend of increasing in SMA and laminin associated with the vasculature mainly in the white matter as illustrated in Fig. 5. Such findings could reflect either attempts at repair of the vessels or the effects of inflammation associated with the immunotherapy (Eng *et al.*, 2004; Scolding *et al.*, 2005; Nicoll *et al.*, 2006). The study was too small to obtain statistically significant differences between the unimmunized and immunized Alzheimer's disease groups with respect to these proteins.

In conclusion, following active Aβ42 immunization in human Alzheimer's disease, we demonstrated that a lower plaque load is associated with an increase in cerebrovascular Aβ. According to the perivascular drainage hypothesis, this may represent Aβ that is being removed from the brain. Specifically, there was a marked increase in Aβ42 in the vessels, consistent with the translocation of

solubilized plaque Aβ42 to the vasculature. The relative lack of both plaques and CAA in the cases with the highest immune response and longest survival time raises the possibility that the process of clearance of Aβ from the brain can progress to completion. Currently, there are ongoing clinical trials of both active and passive immunization for Alzheimer's disease using altered methodology designed to avoid the unwanted inflammatory side effects experienced with the initial trials. Our results suggest that in these new trials there may also be an at least transient increase in CAA as Aβ is cleared from the brain. The effect of the increased CAA on cognitive function remains unknown.

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