

Vaccination with amyloid- β peptide induces autoimmune encephalomyelitis in C57/BL6 mice

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Summary

Recent experimental evidence shows that vaccination with amyloid- β peptide (A β) of transgenic mouse models of Alzheimer's disease protects from the pathological accumulation of amyloid within the CNS. Phase I/II clinical trials of A β vaccination in mild to moderate Alzheimer's disease have been undertaken. Unexpectedly, one of these trials has been suspended because 15 patients showed clinical signs consistent with CNS inflammation. Here, we show that C57BL/6 mice immunized with A β 1–42 peptide develop an inflammatory disease of the CNS characterized by the presence both in the brain and spinal cord of perivenular inflam-

matory foci containing macrophages, T and B cells, and immunoglobulins. The experimental disease was observed only when pertussis toxin, an agent known to favour autoimmune processes, was co-administered. The immune-mediated CNS reaction was associated to A β -induced CD4⁺ cells showing a Th1-type cytokine expression profile and to elevated levels of circulating anti-A β immunoglobulins. Our results indicate that vaccination with A β could determine, under certain circumstances, an aberrant autoimmune-type reaction to A β resulting in a perivenular inflammatory encephalomyelitis.

Keywords: amyloid- β peptide; Alzheimer's disease; encephalomyelitis; vaccination

Abbreviations: A β peptide = amyloid- β peptide; APP = amyloid precursor protein; EAE = experimental autoimmune encephalomyelitis; IFN γ = interferon gamma; IgG = immunoglobulin G; IL-4 = interleukin-4; MOG = myelin oligodendrocyte glycoprotein; p.i. = post-immunization; PT = pertussis toxin

Introduction

The abnormal processing and extracellular deposition of amyloid- β (A β) peptide, a proteolytic derivative of the amyloid precursor protein (APP), leading to the accumulation of fibrils formed from the A β 40–42 amino acid long peptides into amyloid plaques, is a defining characteristic of Alzheimer's disease (for review, see Steiner *et al.*, 1999). Recent evidence suggests that vaccination of transgenic mouse models of Alzheimer's disease with A β causes a marked reduction in brain amyloid burden (Schenk *et al.*, 1999; Janus *et al.*, 2000) and protects these mice from learning and age-related memory deficits (Janus *et al.*, 2000; Morgan *et al.*, 2000). However, the immunological mechanism underlying the protective effect of A β -vaccination in Alzheimer's disease is still not fully determined—apart from the observation that anti-A β antibodies trigger microglial cells to clear amyloid plaques through Fc receptor-mediated phagocytosis (Bard *et al.*, 2000). Based on these results,

phase I/II studies with A β have been initiated in Alzheimer's disease patients with mild to moderate disease (Thattai *et al.*, 2001).

Here we show that vaccination with A β could, in certain circumstances, determine an aberrant autoimmune Th1-type reaction to A β within the CNS, resulting in a perivenular inflammatory encephalomyelitis where macrophages might represent the final effector cells.

Methods

Immunization procedures

Female C57BL/6 mice, 6–8 weeks old, were immunized with incomplete Freund's adjuvant (Sigma, St Louis, MO, USA) supplemented with 4 mg/ml mycobacterium tuberculosis (strain H37Ra; Difco, Detroit, MI, USA) [complete Freund's adjuvant (CFA)], and 100 μ g of A β 1–42 peptide (American

Table 1 Disease features of C57BL/6 mice immunized with CFA only and with myelin oligodendrocyte glycoprotein (MOG)35-55 or β -amyloid 1-42/42-1 with or without PT

Antigen*	Dose of antigen	PT	Disease incidence (%)	Disease onset (range)	Mean maximum neurological score (range)	Cumulative disease score [†]	Mean CNS pathological score (range) [‡]	
							CD3 ⁺ cells	Macrophages
A β 1-42	100 μ g	Yes	16/18 (89)	15.6 \pm 0.6 [§] (13–20)	1.7 \pm 0.3(0–3.0)	47.6 \pm 11.2	6.14 \pm 1.4(0–40)	7.21 \pm 1.2(0–40)
A β 1-42	100 μ g	No	1/10 [¶] (10)	20 (20)	0.1 \pm 0.1(0–1)	0.3 \pm 0.3 [¶]	1.22 \pm 1.1(0–15)	1.25 \pm 0.8(0–10)
A β 42-1	150 μ g	Yes	0/3 [¶] (0)	0 (0)	0(0)	0	–	–
CFA	–	Yes	1/7 (14)	21 (21)	0.1 \pm 0.1(0–1)	0	1.25 \pm 0.7(0–15)	7.39 \pm 1.6(0–30)
MOG35-55	200 μ g	Yes	10/10 (100)	14.1 \pm 0.6 (11–18)	3.7 \pm 0.5(1–6.0)	86.2 \pm 10.8	19.8 \pm 2.4(0–40)	12.2 \pm 2.0(0–40)

*Immunization was performed by subcutaneous injection in the flanks of 300 μ l of an emulsion containing CFA, 4 mg/ml heat-inactivated mycobacterium tuberculosis and the antigen. PT was used or not as indicated. [†]The cumulative disease score is calculated by summing the neurological scores recorded daily during the follow-up. [‡]CNS pathological score was calculated on an average of 12 CNS tissue section per mouse and expressed as mean \pm SE. [§]Numbers are means \pm SE. [¶] $P < 0.005$ versus mice immunized with A β 1-42 and PT (χ^2 test). [¶] $P < 0.001$ versus mice immunized with A β 1-42 and PT (Mann–Whitney for unpaired data). $P < 0.05$ versus mice immunized with A β 1-42 and PT (Student's *t*-Test for unpaired data).

Peptide Company, Sunnyvale, CA, USA). Immunization with A β 1–42 peptide was followed or not by intravenous administration of 500 ng of pertussis toxin (PT) (Sigma) the same day and 48 h later. Mice immunized with 150 μ g of A β 42–1 (BioSource International, Camarillo, CA, USA), 200 μ g of myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (Multiple Peptide Systems, San Diego, CA, USA) or CFA alone, served as controls. Clinical score was recorded daily as follows: 0 = healthy; 1 = transient symptoms suggestive of tail paresis and/or ataxic gait lasting <3 days; 2 = persistent symptoms indicating tail paralysis and gait disturbances lasting >3 days; 3 = paresis of hind-limbs, 4 = paralysis of hind-limbs and/or paresis of forelimbs, 5 = tetra-paralysis, 6 = moribund or death.

All procedures involving animals were performed according to the guidelines of The Animal Ethical Committee of our Institute (IACUC).

Immunological assays

Draining lymph nodes were removed and 4×10^5 lymph node cells per well were cultured in 96-well culture plates (Costar, Cambridge, MA, USA) in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME, USA) supplemented with 2 mM L-glutamine and 50 mg/ml gentamicin (Sigma) and serial concentrations (0.3–10 μ M) of A β 1–42 or MOG35–55. For the T cell proliferation assay, cultures were incubated for 3 days and subsequently pulsed 8 h before harvesting with 1 μ Ci [³H]thymidine (40 Ci/nmol, The Radiochemical Centre, Amersham, UK). Incorporation of [³H]thymidine was measured by liquid scintillation spectrometry.

Intracytoplasmic staining for interferon gamma (IFN γ) and interleukin-4 (IL-4) production was performed on lymph node cells cultured with 10 μ M of A β 1–42 or MOG35–55. After culture, living cells separated on a gradient (Lymphoprep, Axis-Shield, Oslo, Norway) were

re-stimulated with 1 μ g phorbol myristate acetate (PMA, Sigma) and 50 ng ionomycin (Sigma) for 4 h at 37°C, in the presence of 10 μ g/ml brefeldin A (Novartis, Basel, Switzerland) to prevent egress of newly synthesized proteins from the endoplasmic reticulum. After fixing with 4% paraformaldehyde for 20 min at room temperature, cells were stained for CD4/CD8 and IFN γ /IL-4 as previously described (Openshaw *et al.*, 1995).

Anti-A β 1–42 immunoglobulin G (IgG), IgG1 and IgG2a were detected using biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA) antibodies using a previously described home-made enzyme-linked immunosorbent assay (ELISA) (Moiola *et al.*, 1998).

Neuropathological features in experimental autoimmune encephalomyelitis (EAE) mice

At the time of sacrifice, mice were perfused with 4% paraformaldehyde and the brain, spinal cord, spleen, liver, lungs, heart, gastrointestinal tract, kidneys, muscles and skin were removed and embedded in paraffin. Four μ m microtome sections were cut from all organs and stained with haematoxylin and eosin or processed for immunohistochemistry. Luxol Fast Blue staining was performed on brain and spinal cord tissue sections. The following antibodies were used: rat anti-mouse CD3 (pan-T cell marker; Serotec Ltd, Oxford, UK), rat anti-CD45R antibody (B cell marker; B220, Pharmingen, San Diego, CA, USA) and tetramethyl rhodamine isothiocyanate (TRITC) conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Macrophages were stained with biotin-conjugated BS-I isolectin B4 (Sigma). Appropriate secondary antibodies were used when needed.

CNS damage was scored as follows: 10 = traces of leptomeningeal or subpial inflammation; 20 = mild leptomeningeal or subpial inflammation with scarce or no perivascular inflammatory cell aggregates; 30 = moderate leptomeningeal or subpial inflammation with presence of perivascular inflammatory cell aggregates; 40 = leptomeningeal and subpial inflammation with presence of inflammatory cell aggregates in the perivascular space and in the CNS parenchyma.

Results

We immunized C57BL/6 mice with 100 μ g of A β 1–42 (hereafter referred as A β), adopting the same schedule and dosing as described previously for the A β vaccination protocol (Schenk *et al.*, 1999). To boost the T cell immune response against A β , we also studied, in parallel, an additional group of five C57BL/6 mice immunized with 100 μ g of A β and injected intravenously twice with 500 ng of PT (on the day of the immunization and 2 days later). PT was used because of its ability to increase predisposition to autoimmune diseases, possibly inhibiting tolerance mechanisms that normally operate within secondary lymphoid tissues (Cyster *et al.*, 1995).

As expected, none of the mice immunized with A β alone showed any abnormality up to 60 days after immunization. Conversely, all five mice immunized with A β and PT developed symptoms and signs evocative of a neurological disorder affecting the CNS between 13 and 20 days post-immunization (p.i.) (Table 1). Symptoms and signs lasted for up to 75 days, with a caudo-cranial progression and a chronic course, making them indistinguishable from those observed in chronic forms of mouse EAE.

To confirm our preliminary data and to exclude a 'non-specific' CNS effect, 13 additional mice were immunized with 100 μ g A β and 1 μ g PT, and five mice were immunized with 100 μ g A β but not PT. Three mice immunized with 150 μ g of the reverse A β 42–1 peptide and 1 μ g of PT, and seven mice immunized with CFA and 1 μ g of PT served as additional controls. The results are summarized in Table 1. A total of 89% (16 out of 18) of the mice immunized with A β and PT developed a CNS disease, while only 10% (1 out of 10) of the mice immunized with A β alone and 10% (1 out of 10) of the control mice showed minimal clinical symptoms (see Table 1). All 'positive' control C57BL/6 mice immunized with 200 μ g of MOG35–55 and 1 μ g of PT developed the classical chronic-progressive form of EAE (Furlan *et al.*, 2001).

In all mice treated with A β and PT, we found inflammatory aggregates of macrophages and T cells, surrounding small venules within the leptomeningeal space (Fig. 1A) and the brain and spinal cord parenchyma (Fig. 1B and C). Focal inflammatory aggregates were also seen inside the brain and spinal cord parenchyma (Fig. 1F–J). Limited areas of demyelination and myelin debris-containing macrophages were seen around some aggregates (Fig. 1D and E). However,

demyelination was not widespread within the CNS compared with EAE mice (Furlan *et al.*, 2001) and was confined to the subpial and the perivascular space. Inflammatory CNS infiltrates appeared 20 days p.i. and were still present at 75 days p.i. These results support a major role for T cells and macrophages in the pathogenesis of this encephalomyelitis.

We then analysed mice immunized with CFA and PT only. We found macrophages, but not T cells, infiltrating the leptomeningeal space and, occasionally, the perivascular space (Table 1). This latter finding, possibly due to PT injection, may explain the transient mild neurological signs lasting <3 days that we have rarely seen in control mice. In six out of 14 mice co-treated with A β and PT and sacrificed between 60 and 75 days p.i., but not in control mice or MOG35–55 immunized mice, we observed spotty necrotic foci in the spleen and diffuse perivenular neutrophilic aggregates in the connective tissues of the gastrointestinal tract (data not shown).

Since these results were suggestive of a T cell-mediated inflammatory autoimmune disease of the CNS, we analysed the T cell response against A β . In draining lymph nodes of mice treated with A β and PT, we found a clear dose-dependent T-cell response against A β (Fig. 2A). This response was of a Th1-type as indicated by the significant increase of the percentage of A β -specific CD4⁺ T cells producing IFN γ (Fig. 2B). CD4⁺ cells producing IL-4 and CD8⁺ cells producing either IL-4 or IFN γ were virtually absent (Fig. 2B and C).

Next, we analysed the B cell response against A β . As expected, anti-A β IgG antibodies were found in serum samples of mice treated with A β irrespective of PT administration (Fig. 3A). Interestingly, anti-A β IgG appeared earlier in mice treated with A β alone (10 days p.i.) compared with mice co-treated with A β and PT (30 days p.i.) and persisted, in both groups, up to 75 days p.i. We then measured anti-A β IgG subclasses. We found a predominant IgG2a (Fig. 3B) versus IgG1 (Fig. 3C) anti-A β response. Anti-A β IgG1 was at a very low level and was detectable only 30 days p.i. (Fig. 3C). Conversely, IgG2a appeared at day 10 p.i. and increased up to 75 days p.i. (Fig. 3B).

Since Th1-derived IgG2a antibody are complement fixing and participate in macrophage activation and opsonization, we analysed CNS tissue samples from mice co-treated with A β and PT to analyse B cells and IgG deposition in areas of chronic inflammation. We found a consistent number of B cells infiltrating the perivenular spaces of the CNS (Fig. 3D and E) as well as deposits of IgG in the same areas (Fig. 3F).

Discussion

Our results indicate that vaccination with A β may trigger an aberrant autoimmune reaction against A β leading to a perivenular inflammatory encephalomyelitis. This reaction is associated with Th1-like A β -specific T cells, B cells producing complement-fixing anti-A β antibodies and

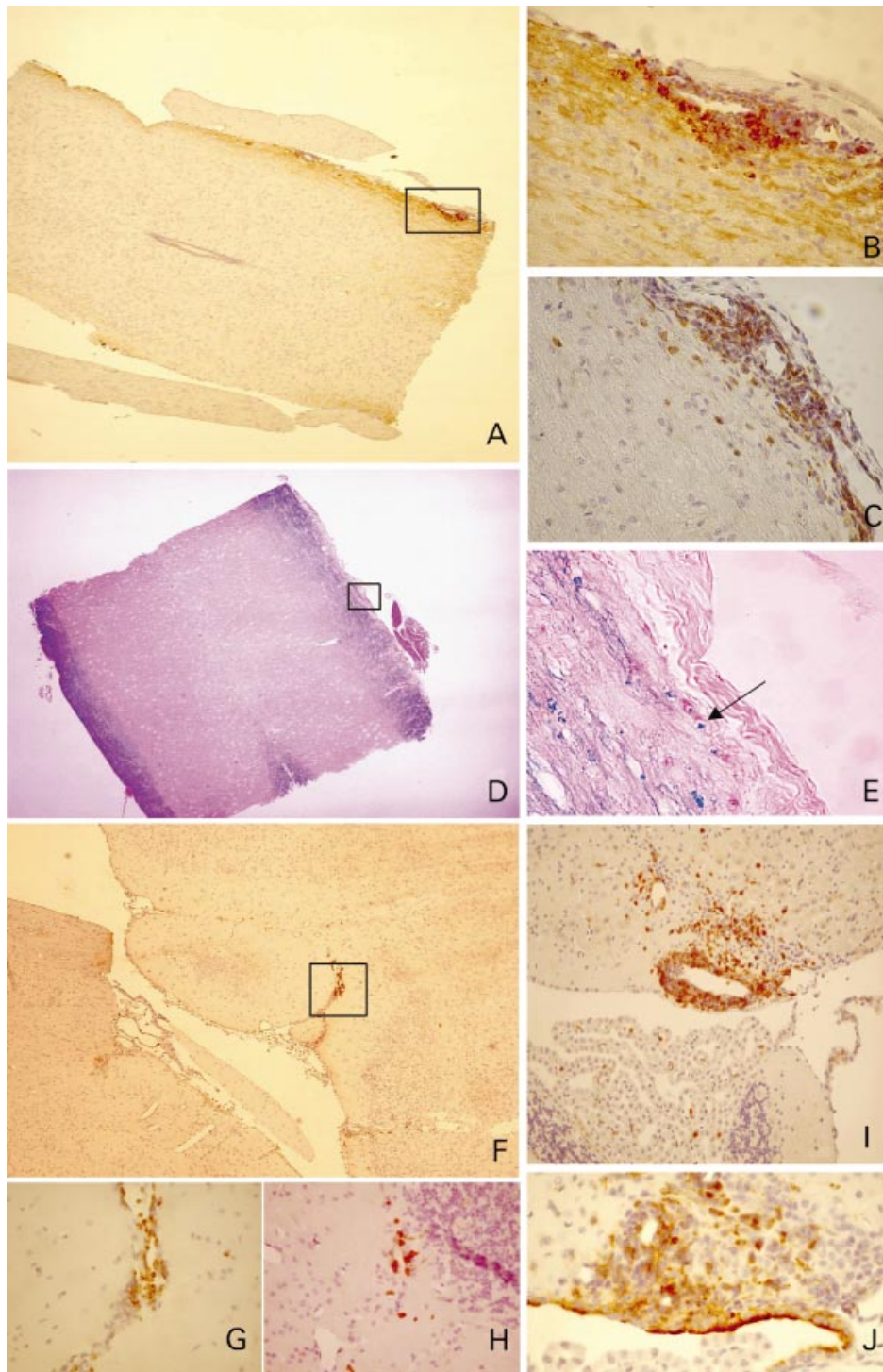


Fig. 1 Pathological findings in the CNS of C57BL/6 mice treated with A β and PT. (A) Macrophage staining showing perivascular aggregates of cells within the leptomeningeal space surrounding the posterior but not the anterior spinal cord column ($\times 2.5$). (B) Magnification ($\times 25$) of the box in panel A. (C) CD3⁺ cells forming a perivascular cuffing around spinal cord leptomeningeal vessels ($\times 25$). (D) Luxol Fast Blue staining of the spinal cord showing perivascular and subpial demyelination in the posterior but not in the anterior column ($\times 2.5$). (E) Magnification ($\times 25$) of the box in panel D showing macrophages containing myelin debris (arrow). (F) Macrophage staining of a brain coronal section ($\times 2.5$). (G) Macrophage staining showing a perivascular aggregate of cells in the brain area identified by the box in panel F ($\times 25$). (H and I) Perivascular aggregates of CD3⁺ cells in the forebrain (H) and in the cerebellum (I) ($\times 25$). (J) Perivascular aggregate of isolectin-positive macrophages in the same cerebellar area as in panel I ($\times 25$).

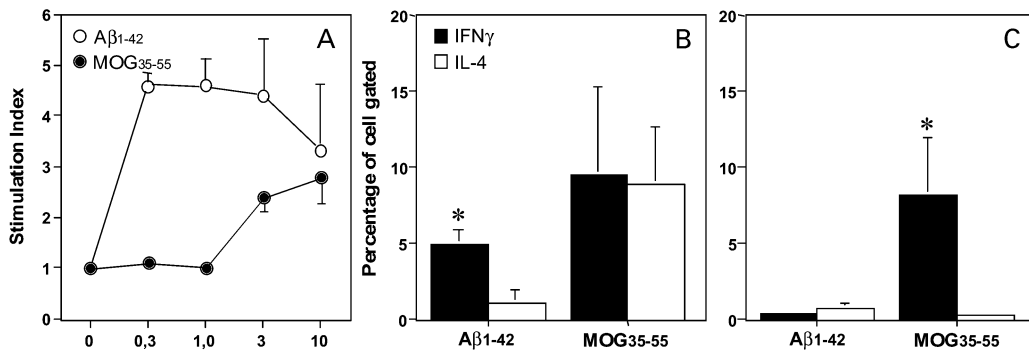


Fig. 2 T cell response in C57BL/6 mice immunized with A β or MOG35-55 and PT. (A) Dose-response curve showing A β - and MOG35-55-induced T cells proliferation. Different amounts of the antigen (from 0.3 to 10 μ M) have been used. Stimulation index is considered positive when >2 . (B) Percentages of CD4⁺ producing IFN γ and IL-4. CD4⁺ cells producing IFN γ are significantly increased in A β -treated mice compared with IL-4-producing CD4⁺ cells. (C) Percentages of CD8⁺ producing IFN γ and IL-4. CD8⁺ cells producing IFN γ are significantly increased in MOG35-55 treated mice, but not A β -treated mice. * $P < 0.05$, Student's *t*-test for unpaired data.

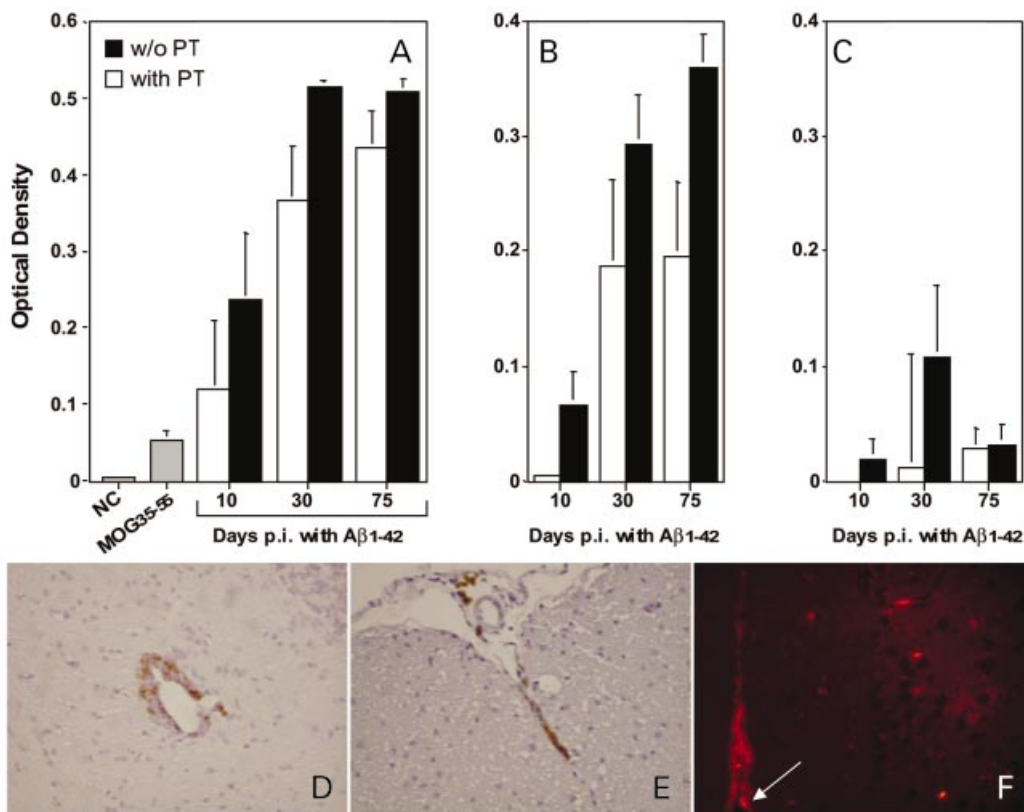


Fig. 3 B cell response in C57BL/6 mice immunized with A β and PT. (A) Anti-A β total IgG levels 10, 30 and 75 days p.i. with A β and PT (open bars) or with A β without PT (filled bars). MOG35-55 and naive C57BL/6 mice (NC) were used as controls (grey bars). (B) Anti-A β IgG2a levels in A β -treated mice at the same time points as in A. (C) Anti-A β IgG1 levels in A β -treated mice at the same time points as in A. (D) B cell immunostaining (CD45R) showing a perivascular aggregate of B cells in the brain of a representative C57BL/6 mice immunized with A β and PT ($\times 40$). (E) B cell immunostaining (CD45R) showing, in the same mice as in D, a perivascular aggregate of B cells in the spinal cord. (F) IgG immunostaining indicating IgG deposition in the spinal cord ($\times 20$). A plasma cell-like cell is indicated within an IgG deposit (arrow).

macrophages, which possibly represent the final effector cells.

The key role of PT in the induction of this CNS-confined autoimmune reaction is clearly indicated by the lack of any abnormal sign or symptom in the CNS of C57BL/6 mice that did not receive PT. The use of PT explains the discrepancy between our results and those indicating that A β vaccination (without PT) induced a protective immune response against A β in transgenic mouse models of Alzheimer's disease (Schenk *et al.*, 1999; Janus *et al.*, 2000; Morgan *et al.*, 2000). However, the occurrence of a CNS-confined autoimmune reaction against a ubiquitous protein, such as A β (Kang *et al.*, 1987) only when PT is co-administered, remains a challenging phenomenon. Some likely explanations can be put forward. PT favours CNS trafficking of blood-borne mononuclear cells by increasing the vascular permeability of cerebral endothelium (Linthicum and Frelinger, 1982; Linthicum *et al.*, 1982). This facilitates CNS-confined (auto)immune reactions, as indicated by the spontaneous occurrence of EAE in T cell receptor transgenic mice for myelin basic protein immunized with PT (Goverman *et al.*, 1993, 1997). Furthermore, CNS vessels are a preferential location for the pathological accumulation of amyloid, possibly owing to a site-specific abnormal processing of APP (e.g. cerebral amyloid angiopathies) (Suzuki *et al.*, 1994; Thomas *et al.*, 1996). This might favour the site-restricted deposit of a normally sequestered antigen, a process which can, in turn, trigger a T cell-mediated (auto)immune reaction.

In mice vaccinated with A β and PT, we also measured increased levels of circulating complement-fixing anti-A β IgG2a. This finding, along with the presence of B cells and IgGs within demyelinating areas of vaccinated mice, suggest these Th1-type antibodies might contribute to the disease process. This is only in apparent contrast with the protective role of anti-A β antibodies that, in transgenic mouse models of Alzheimer's disease, trigger microglial cells to clear amyloid plaques via complement components (Bard *et al.*, 2000; Brazil *et al.*, 2000; Webster *et al.*, 2001). Complement-fixing antibodies may, in fact, be both protective and pathogenic, depending on the circumstances, owing to their opsonising ability (Abbas *et al.*, 1996).

The data reported here support the notion that the use of A β vaccination may lead to an aberrant autoimmune reaction against A β provoking CNS inflammation. This experimental evidence may explain the unexpected appearance of clinical signs consistent with CNS inflammation occurring in 15 patients with Alzheimer's disease undergoing the A β vaccination trial (i.e. AN-1792 trial). Consistent with our data, this adverse event, leading to the definitive suspension of the AN-1792 trial (Birmingham and Frantz, 2002), can be *bona fide* attributed to an (auto)immune mediated process confined to the CNS and triggered by the A β vaccination protocol since the spinal taps from the 15 patients showed elevated protein and lymphocyte levels but absence of bacteria or viruses (<http://www.elan.com/NewsRoom/>).

In conclusion, our work reports a novel observation clearly indicating that a CNS-confined autoimmune reaction against a self-protein such as APP/A β can be induced in mice by a vaccination protocol. Thus, a therapeutic approach based on A β vaccination can be also detrimental and not exclusively protective, as previously suggested in transgenic mouse models of Alzheimer's disease (Schenk *et al.*, 1999; Janus *et al.*, 2000; Morgan *et al.*, 2000). Our data challenge the hypothetical idea to use A β vaccination as a treatment option for Alzheimer's disease.

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