Number of Aβ Inoculations in APP+PS1 Transgenic Mice Influences Antibody Titers, Microglial Activation, and Congophilic Plaque Levels

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ABSTRACT

There have been several reports on the use of β-amyloid (Aβ) vaccination in different mouse models of Alzheimer’s disease (AD) and its effects on pathology and cognitive function. In this report, the histopathologic findings in the APP+PS1 doubly transgenic mouse were compared after three, five, or nine Aβ inoculations. The number of inoculations influenced the effects of vaccination on Congo red levels, microglia activation, and anti-Aβ antibody titers. After three inoculations, the antibody titer of transgenic mice was substantially lower than that found in nontransgenic animals. However, after nine inoculations, the levels were considerably higher in both genotypes and no longer distinguishable statistically. The number of inoculations influenced CD45 expression, an indicator of microglial activation. There was an initial upregulation, which was significant after five inoculations, but by nine inoculations, the extent of microglial activation was equivalent to that in mice given control vaccinations. Along with this increased CD45 expression, there was a correlative reduction in staining by Congo red, which stains compact plaques. When data from the mice from all groups were combined, there was a significant correlation between activation of microglia and Congo red levels, suggesting that microglia play a role in the clearance of compact plaque.

INTRODUCTION

ALZHEIMER’S DISEASE (AD) is a progressive neurodegenerative disorder characterized by accumulation of senile plaques consisting of β-amyloid (Aβ) peptide of which there are two forms, Aβ1-40 and Aβ1-42. This is thought to be the key step in the pathogenesis of AD (Selkoe, 1991). The disorder is also characterized by the formation of neurofibrillary tangles consisting of tau protein and by the initiation and proliferation of a brain-specific inflammatory response (Akiyama et al., 2000). Transgenic mouse models of AD have been an invaluable source of information regarding the pathological progression of the disease and a vehicle in which to test possible therapeutic interventions. Here, we used a transgenic mouse model of AD carrying two transgenes: amyloid precursor protein (APP) and presenilin-1 (PS1), previously described (Duff et al., 1996; Hsiao et al., 1996; Holcomb et al., 1998, 1999; Gordon et al., 2001a, b).

Schenk and associates initially described the effects of Aβ1-42 immunization in the PDAPP mouse. In a report published in 1999, they demonstrated the ability of their vaccination regimen to reduce Aβ deposits in the brain. More recently, Aβ vaccination has also been shown to prevent the cognitive decline in some transgenic mice (including the APP+PS1) in addition to reducing Aβ load (Morgan et al., 2000; Janus et al., 2000). The data presented in this report examine the effect of increasing numbers of Aβ1-42 immunizations on the pathology of the APP+PS1 mouse, specifically, anti-Aβ antibody titers, congophilic plaque load, and activation of microglia. Of particular note was the observed activation of microglial cells, which are central to the inflammatory process in AD, with a concomitant reduction in congophilic plaque.

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MATERIALS AND METHODS

Vaccination protocol

The Tg2576 APP mice (Hsiao et al., 1996) were bred with PS1 line 5.1 mice (Duff et al., 1996) to obtain the double-transgenic mice. These mice were then randomly assigned to groups that received vaccination with either human Aβ1-42 peptide (Bachem) or keyhole limpet hemocyanin (KLH) as described previously (Morgan et al., 2000). Briefly, 100 µg of Aβ1-42 or KLH was dissolved in water at 2.2 mg/ml, mixed with PBS, and incubated overnight. One day later, this suspension was mixed with Freund’s adjuvant (complete for the primary inoculation, incomplete for the next four inoculations) or mineral oil (for the remaining inoculations).

Three vaccination groups were established. The first group received three inoculations starting at an average age of 13 months. These mice were sacrificed at an average of 16 months of age, 13 days after the final inoculation. A second group of mice were given five inoculations starting at an average age of 14.5 months. These mice were sacrificed at an average of 19.75 months of age, 10 days after the last inoculation. A third group was given nine inoculations starting at an average age of 7.5 months. They were sacrificed at an average of 16.25 months of age, 6 weeks after the last inoculation. In addition, nontransgenic mice received either three or nine inoculations of Aβ peptide. Antibody titers were measured by ELISA as described previously (Morgan et al., 2000; Dickey et al., 2001). The KLH-immunized mice are herein referred to as “control mice” for these experiments.

Histopathology examination

Mice were given a lethal overdose of pentobarbital and perfused with 0.9% saline, and the brain was removed. The right side of the brain was rapidly dissected over ice and the left side fixed for 24 h in freshly prepared buffered 4% paraformaldehyde. Following cryoprotection through increasing concentrations of sucrose solutions at 24-h intervals, frozen sections were cut on a sliding microtome at a 25-µm thickness and stored in Dulbecco’s PBS with sodium azide to prevent microbial growth. Sections were stained using floating immunohistochemistry methods for total Aβ (rabbit antiserum primarily reacting with the N terminus of the Aβ peptide; 1:10000) and CD45 (Serotec; 1:3,000) as described previously (Holcomb et al., 1999; Gordon et al., 1997). Sections were also mounted on slides and stained with Congo red (Sigma-Aldrich) (Gordon et al., 2001a). The size of the area of the hippocampus and frontal cortex that stained was measured with a Videometric V150 image analysis system (Oncor) on a Nikon Microphot FX microscope as described in detail previously (Gordon et al., 2001a). The percentage area was measured and analyzed. Data were collected from 8 to 16 equally spaced horizontal sections. The values for all sections from one mouse were averaged to obtain a single sample for statistical analysis.

Statistical analysis

Data were first analyzed by comparing Aβ-vaccinated and control mice within each group by one-way ANOVA using the Statview software program (SAS). Because of differences in the age of sacrifice, the results from each Aβ-vaccinated mouse were normalized to the mean value of their respective control vaccinated group (percent of control) to correct for differences in age at sacrifice and overall staining intensity for each inoculation group. These data were then analyzed by performing a simple regression analysis with the Statview program.

RESULTS

Antibody titers increased with increasing numbers of inoculations in both transgenic and nontransgenic mice (Fig 1). Initially, doubly transgenic mice had lower anti-Aβ titers than similarly treated nontransgenic mice. However, after the ninth vaccination, the transgenic mice produced antibody titers that were similar to those in the nontransgenic mice.

In general, the Congo red staining in the hippocampus was reduced as a result of Aβ vaccination (Fig. 2A, B). In the group receiving five inoculations, this reduction was almost 50% and was significantly greater than in the control mice ($P < 0.002$; Fig. 3). In the groups receiving three or nine inoculations, the reduction in the Congo red-stained area was not statistically significant. The results from the frontal cortex also followed these trends, although no significant difference was found at any time points (Table 1).

Expression of CD45 was increased almost twofold in the hippocampus of mice given five inoculations (Fig. 2C, D; Fig. 4; $P < 0.001$). There was a similar trend toward CD45 upregulation in the group receiving three inoculations, although the difference was not statistically significant. However, the vaccinated mice were equivalent to the control mice in the group receiving nine inoculations. The same trend was seen in the frontal cortex, although not to the same degree (Table 1).

When data from all three groups are combined, there was a correlation between CD45 expression and Congo red staining...
in the hippocampus, as illustrated in Figure 5. This relation shows that mice with elevated CD45 expression had less Congo red staining ($r = 0.691; P = 0.002$). This result did not occur because of a biased positioning of one of the inoculation groups. By plotting the data from each group with a different symbol, one can see that in each inoculation group, there are mice with high CD45 staining and other mice with little, which was less than the control average. This bimodal distribution of CD45 staining has been observed in most groups of mice we have examined, including the control mice in the present study.

**DISCUSSION**

Expression of CD45 is indicative of microglial activation. Here, we have shown that CD45 expression in transgenic mice receiving Aβ vaccination is upregulated after five inoculations. However, on average, the expression was no longer elevated after a ninth inoculation. This result suggests that the microglial activation resulting from Aβ vaccination is transient. This likely represents a desensitization to the circulating antibodies, be-

**FIG. 2.** Histologic effects of immunization. (A, B) Congo red staining of hippocampus of the mice receiving five Aβ inoculations (original magnification ×40). (C, D) Staining of CD45, counterstaining with Congo red, in the hippocampus of the mice receiving five Aβ inoculations (original magnification ×200). Panels A and C are from KLH-immunized mice; panels B and D are from Aβ-immunized mice. A, B: scale bar = 250 μm; C, D: scale bar = 50 μm.

**FIG. 3.** Congo red levels in hippocampus relative to number of inoculations for vaccinated and control APP + PS1 mice. All mice for each group were averaged. **P < 0.002.
cause even though the interval between the last inoculation and sacrifice after nine inoculations was 6 weeks, the antibody titers were still high at necropsy.

Perhaps the most interesting data were the high correlation between Congo red staining levels in the hippocampi of transgenic mice and extent of activation of microglia. These data add to a growing body of literature suggesting that in transgenic mice, activation of microglia leads to clearance of Aβ deposits.

Ongoing work from our group shows reduction in amyloid deposits in association with microglial activation in several circumstances using the transgenic mouse model of amyloid deposition. Administration of a flurbiprofen derivative (NCX-2216) that slowly releases nitric oxide causes dramatic activation of microglia and substantial reduction in Congo red staining (Jantzen et al., in press). In another study, intrahippocampal injections of lipopolysaccharide, a pro-inflammatory agent, simultaneously reduced the Aβ load and activated microglia (Di Carlo et al., 2001). Even in the normal time course of amyloid accumulation, there is a stabilization of the congophilic deposits in doubly transgenic mice between 12 and 15 months, the age at which microglial activation becomes most pronounced (Gordon et al., 2001b).

It has been well demonstrated that microglia in culture are readily capable of internalizing Aβ1-42 aggregates (Paresce et al., 1996; Webster et al., 2001). The data reported here are consistent with several other reports regarding Aβ vaccination and microglial activation. In the original Aβ vaccination report, the vaccine was found to result in activated microglia around the few deposits that did remain (Schenk et al., 1999). Bard and associates (2000) also demonstrated that microglia can be activated to clear tissue amyloid deposits by Fc receptor-mediated phagocytosis in vitro. In a direct experiment, Bacskai and colleagues (2001) demonstrated that injection of anti-Aβ antibodies into transgenic mouse brain induced a rapid disappearance of Aβ associated with a florid microglial reaction. These results, together with those from our research group, indicate that activation of microglia can have benefit in clearing Aβ deposits from the brains of transgenic mice. It is unclear whether excessive activation of microglia can ultimately cause autotoxic inflammatory reactions in this model or whether the mouse brain is somehow resistant to the development of such a reaction. It is intriguing that the microglial activation had largely subsided in mice receiving nine inoculations. This finding suggests that under these circumstances, the microglia can develop tolerance to the activating stimuli. If so, vaccination may be one mechanism to, perhaps paradoxically, reduce an autotoxic reaction in the AD brain.

The observation that the doubly transgenic mice are slower to mount an immune response after Aβ vaccination than their...
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nontransgenic counterparts is a significant one. There are several plausible explanations for this impaired antibody response. One explanation is that Aβ is a “self-antigen” in the transgenic mice (expressing human APP), and thus, they do not mount as significant a humoral response to the injected human Aβ1-42. The murine Aβ sequence is slightly different from the human sequence; thus, nontransgenic mice would not identify the human Aβ peptide as an autoantigen. Another possibility that the transgenic mice are by some means immune compromised, as is seen in older humans; thus, they are slower to mount a significant immune response. A third explanation is absorption of the serum antibodies by circulating Aβ, which interferes with antibody binding to the ELISA plate. This effect might be most evident when the antibody titers are low and antibody concentrations are stochiometrically similar to that of Aβ. In any event, repeated vaccinations ultimately overcome this restriction of antibody generation in the transgenic mice. This may have significance for the treatment of human populations, with multiple vaccinations required to activate a vigorous antibody response.

In conclusion, the data here are consistent with the argument that Aβ vaccination results in plaque clearance primarily through activation of microglial cells. Still, we continue to entertain the possibility of antibodies dissolving plaques directly (Solomon et al., 2001) or that antibodies bind circulating Aβ, reducing the effective plasma Aβ concentration and increasing the Aβ concentration gradient between brain and blood, leading to more rapid Aβ removal from the central nervous system. Finally, we believe that multiple inoculations are likely to be required if Aβ vaccination demonstrates efficacy in the treatment or prevention of AD.

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