American Journal of Pathology, Vol. 151, No. 4, October 1997

Short Communication

Amyloid A Protein Amyloidosis Induced in Apolipoprotein-E-Deficient Mice

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Apolipoprotein E (apoE) is a constituent of lipoproteins other than low-density lipoprotein, and it principally acts in the transport and metabolism of plasma cholesterol and triglyceride. ApoE is a minor constituent of various kinds of amyloidoses and may play a role as a pathological chaperone for fibrillogenesis of amyloid fibril protein with the amyloid P component and proteoglycans. In this study, we examined the role of apoE in amyloidogenesis *in vivo* in apoE-deficient mutant mice with amyloid A protein (AA) amyloidosis induced by inflammatory stimulation. Amyloid deposition was seen in six of nine C57BL/6J control mice and in six of eight apoE-deficient mutant mice after the intraperitoneal and subcutaneous injections of the mixture of complete Freund's adjuvant and *Mycobacterium butyricum*. Moreover, amyloid deposition in apoE-deficient mice as well as C57BL/6J control mice started 48 or 72 hours after injection of amyloid-enhancing factor and silver nitrate, although the amount of amyloid deposit in C57BL/6J control mice was slightly larger than that in apoE-deficient mice. These amyloid deposits reacted with anti-mouse AA antibody were seen in the perifollicular area of the spleen. Immunoreactivity of apoE was seen irregularly in the amyloid deposits of C57BL/6J control mice but not in the amyloid deposit of apoE-deficient mice. From these results, we concluded that apoE is not always necessary for amyloid deposition and that the existence of apoE might slightly accelerate AA amyloid deposition in the earliest phase of AA amyloid deposition. *(Am J Pathol 1997, 151:911–917)*

Amyloidosis is a disease caused by amyloid deposition in various tissues and organs. Amyloid fibril protein is a major constituent of amyloid deposit, and at least 17 types of amyloid fibril proteins have been found.[1–3] The amyloid P component,[4] proteoglycans,[5] and apolipoprotein E (apoE)[6] are commonly associated with amyloid deposits as minor components regardless of the type of amyloidosis. These minor components may play a role as a pathological chaperone in fibrillogenesis for the amyloid fibril proteins.[6] ApoE is a constituent of lipoproteins other than low-density lipoprotein, and it principally acts in the transport and metabolism of plasma cholesterol and triglyceride.[7,8] Several studies have demonstrated the immunoreactivity of apoE in senile plaque in Alzheimer's disease, amyloid deposits in various types of systemic amyloidoses including murine amyloid A protein (AA) amyloidosis, and localized islet amyloid.[6,9–13] Furthermore, it has been reported that apoE4, which is one of three major isoforms of apoE, is a risk factor for Alzheimer's disease.[14]

AA amyloidosis is one representative type of systemic amyloidosis in humans and in mice. AA amyloidosis can be induced rapidly in mice by inflammatory stimulation using either a mixture of complete Freund's adjuvant and *Mycobacterium butyricum*[15] or amyloid-enhancing factor (AEF) and silver nitrate ($AgNO_3$).[16] Recently, Maeda and colleagues generated apoE-deficient mutant mice by gene targeting[17] and used them for atherogenic study.[18] In this study, we tried to induce AA amyloidosis in apoE-deficient mutant mice by inflammatory stimulation to evaluate the role of apoE in amyloidogenesis *in vivo*.

Materials and Methods

ApoE-deficient mutant mice (C57BL/6J-Apoe^{m1Unc})[17] and C57BL/6J control mice were purchased from Jack-

Supported in part by a grant from the Amyloidosis Research Committee, Surveys and Research on Specific Diseases, the Ministry of Health and Welfare of Japan.

Accepted for publication July 23, 1997.

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son Laboratory, Bar Harbor, ME. For the first experimental design, AA amyloidosis was induced in each strain of mice according to the method of Ram et al[15] with some modifications. The mixture (0.2 ml) of complete Freund's adjuvant and *M. butyricum* was injected intraperitoneally in each mouse. A second injection of the mixture (0.2 ml) of complete Freund's adjuvant and *M. butyricum* was given subcutaneously after 1 week. Nine C57BL/6J mice and eight apoE-deficient mutant mice were sacrificed 2 weeks after the second injection, and sera and spleens were obtained from each mouse.

For the second experiment, AA amyloidosis was induced in mice by injecting AEF and $AgNO_3$ to evaluate the earliest stage of amyloid deposition. AEF was prepared from apoE-deficient mice according to the method of Axelrad et al[16] with some modifications as follows: the mixture (0.25 ml) of complete Freund's adjuvant and *M. butyricum* was injected twice in apoE-deficient mice subcutaneously at 1-week intervals. These mice were sacrificed 6 weeks after the second injection. Livers of these mice were homogenized with 8 ml of 4 mol/L glycerol, 0.01 mol/L Tris/HCl (pH 7.6) per gram of tissue. The homogenates were shaken for 1 hour at 4°C and then centrifuged at 20,000 × *g* for 1 hour at 4°C. The supernatant was dialyzed overnight against phosphate-buffered saline in a Spectra/Por 3 Molecularporous dialysis membrane (Spectrum, Houston, TX) with a molecular weight cutoff of 3500. In each mouse, 0.5 ml of AEF was administered intraperitoneally, and 0.5 ml of 2% $AgNO_3$ was administered subcutaneously. Ten mice of each strain were sacrificed 48 hours after administration of AEF and $AgNO_3$. Ten C57BL/6J mice and nine apoE-deficient mutant mice were sacrificed 72 hours after administration of AEF and $AgNO_3$. Sera and spleens were obtained from each mouse. Sera were stored at −80°C until biochemical analysis. Spleens were fixed in 10% formalin and embedded in paraffin for histological and immunohistochemical examination.

Histological and Immunohistochemical Examination

Sections were cut 4 μm thick from paraffin-embedded blocks of the spleen, stained with alkaline Congo red, and examined under polarized light. Immunohistochemical examinations were performed with an indirect immunoperoxidase method. After deparaffinization, the sections were treated with 3% H_2O_2 in methanol for inhibition of endogenous peroxidase and with 10% normal goat serum for inhibition of nonspecific reaction. Rabbit anti-mouse AA[19] (1:2000) for all cases and anti-human apoE (1:100; DAKO, Glostrup, Denmark) for representative cases were applied as primary antibodies for 30 minutes at room temperature. Peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (1:20; DAKO) was applied as secondary antibody for immunohistochemistry. Next, the sections were visualized using diaminobenzidine as a substrate. Antigen retrieval for anti-human apoE antibody was performed in a target retrieval solution (DAKO) for 10 minutes at 98°C using a microwave oven[20] or in 100% formic acid[21] for 1 minute at room temperature after deparaffinization. As a positive control for immunostaining of anti-human apoE antibody, we used the formalin-fixed, paraffin-embedded sections of the kidney and liver involved in murine AApoAII amyloidosis, which had clearly reacted with the antibody in the preliminary study (data not shown). The amino acid sequence homology is 70% between human apoE and murine apoE[22]; therefore, polyclonal anti-human apoE antibody is likely to react with murine apoE.

Immunoblotting of ApoE

The sera of two C57BL/6J and two apoE-deficient mice, in which amyloid deposits were histologically confirmed in the spleen, were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subsequent immunoblotting to confirm whether these mice have apoE. Each sample was applied on 10% SDS-polyacrylamide gel for electrophoresis and then transblotted on the polyvinylidene difluoride membrane. After blocking of the nonspecific reaction using 3% bovine serum albumin, the membrane was incubated with either rabbit anti-human apoE (1:1000) or normal rabbit IgG (1:1000; DAKO) as primary antibody. The following immunoreaction was performed using a Vectastain ABC standard kit (Vector Laboratories, Burlingame, CA) and visualized using diaminobenzidine as a substrate. As positive control, purified mouse apoE was loaded on the SDS-polyacrylamide gel at the same time. The purified mouse apoE was prepared as follows. Triamcinolone diacetate (0.15 mg/mouse) was injected subcutaneously in 100 ddY mice to increase apoE.[23] Very-low-density lipoprotein ($d < 1.006$ g/ml) was isolated from the sera of these mice by preparative ultracentrifugation and delipidated with ethanol/ethyl ether (3:1) as described previously.[24] Apolipoproteins obtained from very-low-density lipoprotein by delipidation were electrophoresed using SDS-polyacrylamide (11%) gel (10 cm × 10 cm × 3 mm). The band with the molecular weight that corresponded to apoE was cut out from the SDS-polyacrylamide gel and used as purified mouse apoE.

Results

Histological and Immunohistochemical Findings

After the injections of complete Freund's adjuvant and *M. butyricum*, Congo-red-positive amyloid deposits were seen in the perifollicular area of the spleens from six of nine C57BL/6J control mice (Figure 1a) and six of eight apoE-deficient mice (Figure 1b). Forty-eight hours after administration of AEF and $AgNO_3$, 9 of 10 C57BL/6J control mice and 8 of 10 apoE-deficient mice had amyloid deposits in the perifollicular area of the spleens. Similarly, 72 hours after administration of AEF and $AgNO_3$, all controls and apoE-deficient mice showed such deposits. Some mice had a minute amyloid deposit, which was barely confirmed under polarized light, and others had rather large deposits. These amyloid deposits inevitably

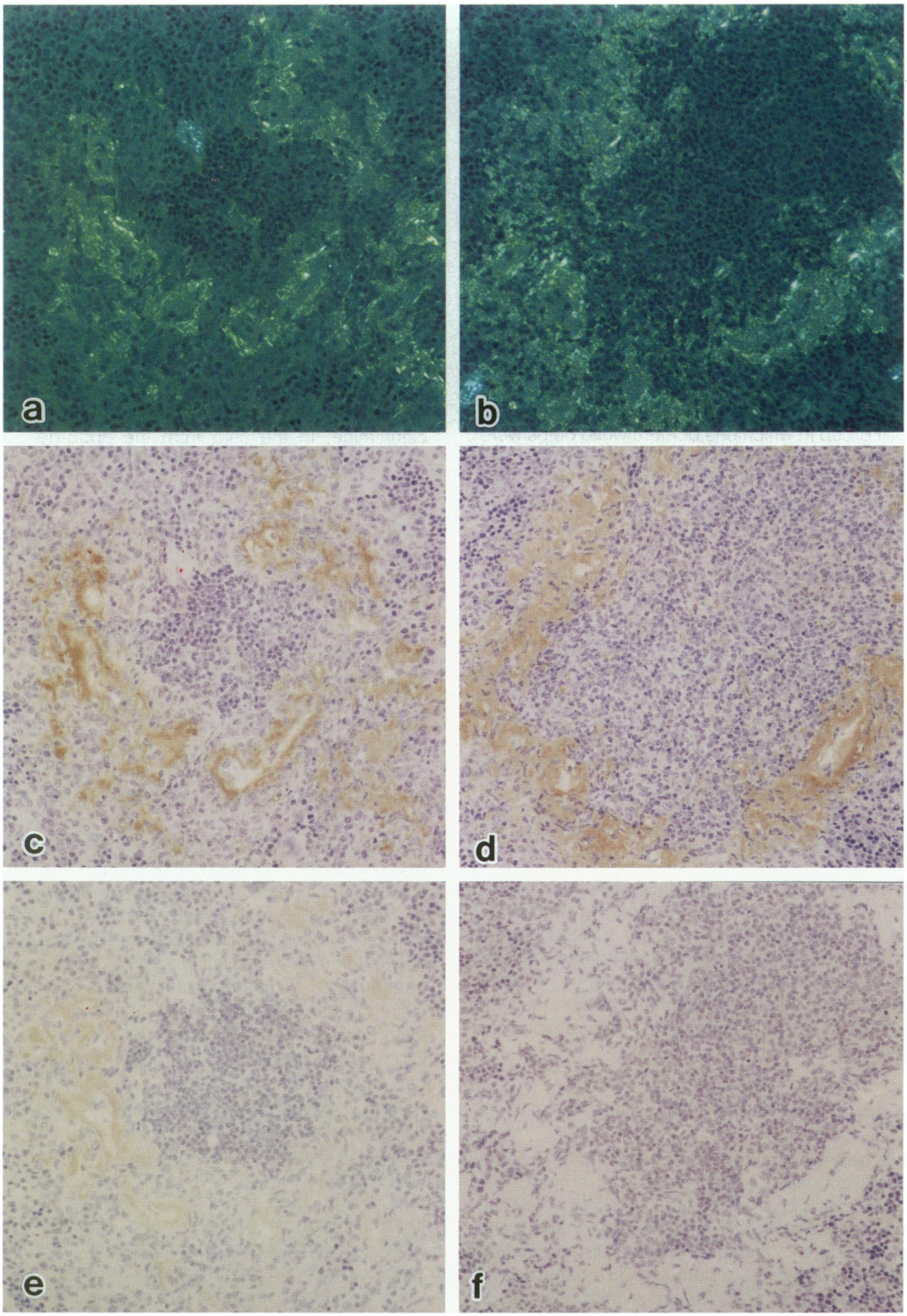

Figure 1. Amyloid deposit in a C57BL/6J control mouse and an apoE-deficient mouse induced by complete Freund's adjuvant and *Mycobacterium butyricum*. A medium amount of amyloid deposit is present in the perifollicular area of the spleen from the C57BL/6J control mouse (**a**, **c**, and **e**) and the apoE-deficient mouse (**b**, **d**, and **f**). These deposits show green birefringence under polarized light with Congo red staining (**a** and **b**). The immunoreactions of anti-mouse AA antibody with amyloid deposits of both strains are approximately homogeneous in intensity (**c** and **d**). An irregular and somewhat weak immunoreaction of anti-human apoE antibody is seen in the amyloid deposit of a C57BL/6J control mouse (**e**); however, no positive reaction is seen in the amyloid deposit of an apoE-deficient mouse (**f**). Original magnification, ×50.

Table 1. Amyloid Deposition in Each Group of Mice

Strain	Induction method	Examined cases	Amyloid deposition		
			Small amount	Medium amount	None
C57BL/6J mice	Adjuvant* + *M. butyricum*	9	3	3	3
	48 hours after AEF + $AgNO_3$	10	9	0	1
	72 hours after AEF + $AgNO_3$	10	3	7	0
ApoE-deficient mice	Adjuvant + *M. butyricum*	8	2	4	2
	48 hours after AEF + $AgNO_3$	10	8	0	2
	72 hours after AEF + $AgNO_3$	9	7	2	0

*Complete Freund's adjuvant.

reacted with anti-mouse AA antibody (Figures 1, c and d, and 2). The amount of amyloid deposit was semiquantitatively classified into two categories: small-deposit group in which no splenic white pulp was surrounded completely by the deposit (Figure 2, a and b) and medium-deposit group in which one or more white pulps were surrounded entirely by the deposit (Figure 2, c and d). Using this criterion, the mice from each strain and within each induction method grouping were classified into the two deposit categories. The distribution is shown in Table 1.

Immunoreactivity for anti-human apoE antibody with amyloid deposits was seen in C57BL/6J mice, but the reaction was irregular and rather weaker than that for anti-mouse AA antibody regardless of antigen retrieval pretreatments (Figure 1e). Amyloid deposits in apoE-deficient mice did not react with anti-human apoE antibody despite differences in antigen retrieval pretreatments (Figure 1f). Anti-human apoE antibody reacted strongly with the amyloid deposits of AApoAII amyloidosis that were used as a positive control.

Immunoblotting of ApoE

Anti-human apoE antibody reacted intensely with the approximately 34-kd band in the sera from two C57BL/6J mice and the mouse apoE purified for positive control (Figure 3). This band was not seen in the sera from two apoE-deficient mice. Some bands higher than 45 kd were stained weakly with anti-human apoE antibody in the sera from all mice, but these bands were also stained by normal rabbit IgG.

Discussion

There have been several reports that apoE is associated with amyloid deposits in various types of amyloidosis, such as senile plaque in Alzheimer's disease, systemic amyloidosis including murine AA amyloidosis, and localized islet amyloid.[6,9–13] Some investigators suggested that apoE may serve as a pathological chaperone in amyloid fibrillogenesis.[6,11] However, certain kinds of amyloid precursor proteins, such as β_2-microglobulin[25] and transthyretin,[26] form amyloid-like fibrils under the condition of apoE deficiency *in vitro*. Whether apoE is essential in amyloid deposition *in vivo* has been unclear. In the present study, amyloid deposition occurred in apoE-deficient mice as well as in C57BL/6J control mice after inflammatory stimulation. This amyloidosis was confirmed as AA type by immunohistochemical examination using anti-mouse AA antibody. No apoE immunoreactivity was detected in the amyloid deposits of apoE-deficient mice. In the sera from the apoE-deficient mice associated with amyloid deposition, apoE was not detected by immunoblotting using anti-human apoE antibody, which did recognize apoE in the sera from the C57BL/6J control mice and mouse apoE purified for positive control. From these findings, we concluded that apoE is not always necessary for the formation of murine AA amyloid deposits.

The immunoreaction of AA amyloid deposits with anti-human apoE antibody was irregular and somewhat weak in intensity in C57BL/6J control mice, although the amyloid deposits reacted almost homogeneously with anti-mouse AA antibody. From these findings, we assume that apoE is unevenly distributed among AA amyloid deposits. However, the possibility that the antigenicity of apoE partially decreased during the tissue processing cannot be excluded.

The reason that apoE is associated with amyloid deposits regardless of the type of amyloid fibril protein has not been elucidated in this study. Reportedly, amyloid β-protein is able to bind apoE *in vitro*[27,28]; thus, apoE may be associated with amyloid deposits by binding with the amyloid fibril protein itself in some types of amyloidoses. Furthermore, apoE has been shown to bind heparan sulfate proteoglycan,[8,29] which is also an amyloid-associated protein. Thus, apoE may be associated with amyloid deposits by binding with heparan sulfate proteoglycan located in the deposits.

ApoE, especially apoE4, is thought to play a role in the pathogenesis of Alzheimer's disease, and clinically, apoE4 is designated as a risk factor of Alzheimer's disease.[14] Regarding the role of apoE in the fibrillogenesis of β-protein, apoE, especially apoE4, accelerates *in vitro* fibril formation of Aβ1–40[27] and Aβ1–42.[28] However, there is a contradictory finding that all three isotypes of apoE (E2, E3, and E4) inhibit fibril extension of Aβ1–40 *in vitro*.[30] Ishii et al reported that dosage of apoE ϵ4 allele correlated significantly with cortical Aβ1–40 levels but not with Aβ1–42 levels in sporadic Alzheimer's disease.[31] Therefore, whether apoE, especially apoE4, accelerates β-protein deposition *in vivo* remains controversial. Some investigators have proposed that other

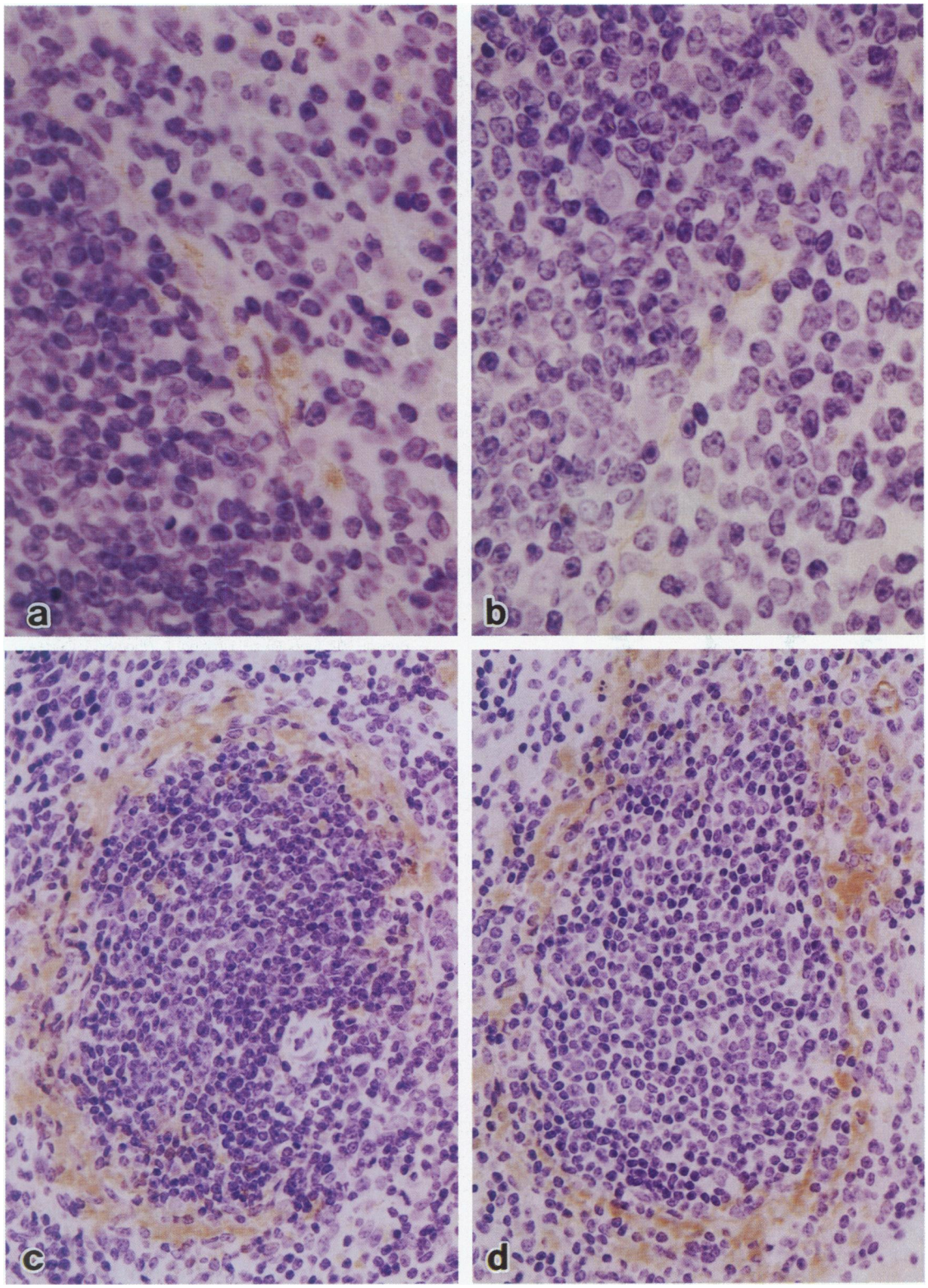

Figure 2. Various amounts of amyloid deposition appear in C57BL/6J control mice and apoE-deficient mice sacrificed 72 hours after administration of amyloid-enhancing factor and silver nitrate. A small amount of amyloid deposit is seen in the perifollicular area of the spleen in a C57BL/6J control mouse (**a**) and an apoE-deficient mouse (**b**) by immunostaining with anti-mouse AA antibody. Original magnification, ×200. A medium amount of amyloid deposit, in which follicle is almost entirely surrounded, is also seen in the spleen of another C57BL/6J control mouse (**c**) and another apoE-deficient mouse (**d**) by immunostaining with anti-mouse AA antibody. Original magnification, ×100.

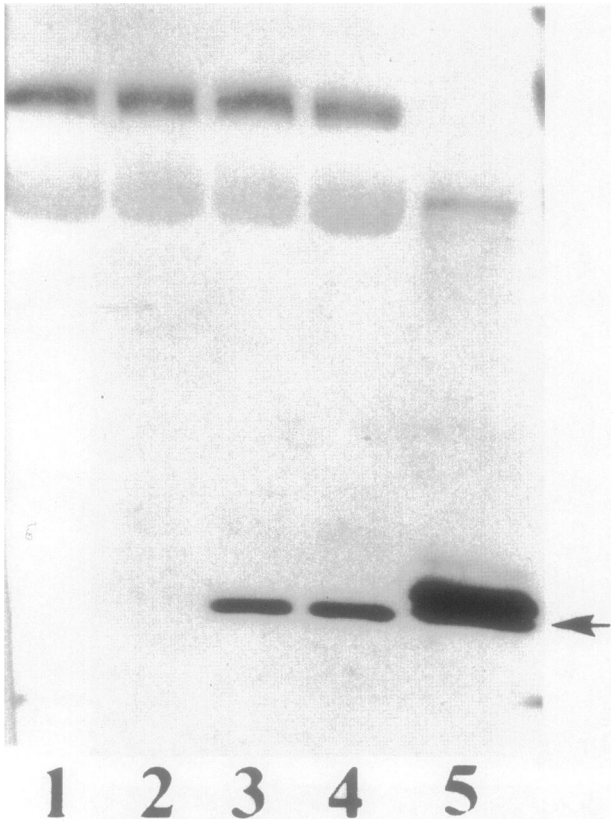

Figure 3. Immunoblotting of apoE using anti-human apoE antibody. **Lanes 1** and **2**, apoE-deficient mice; **lanes 3** and **4**, C57BL/6J control mice; **lane 5**, purified mouse apoE. Anti-human apoE antibody reacts with an approximately 34-kd band in the sera from C57BL/6J control mice and purified mouse apoE (**arrow, lanes 3, 4,** and **5**). This band is not seen in the sera from apoE-deficient mice (**lanes 1** and **2**).

mechanisms of apoE, rather than β-protein deposition, may contribute to the pathogenesis of Alzheimer's disease.[32,33] Nathan et al reported that, in the presence of β-migrating very-low-density lipoproteins, apoE4 decreased neurite outgrowth whereas apoE3 increased it.[32] Crutcher et al documented that a 22-kd fragment derived from apoE is neurotoxic and that the fragment from apoE4 is significantly more toxic than that from apoE3.[33] Thus, the role of apoE in the pathogenesis of Alzheimer's disease may be more complex than its role in other amyloidoses.

As it is well known that AA amyloid deposition starts within 48 hours after injection of AEF and $AgNO_3$,[16] we performed a rapid amyloid induction method using administration of AEF extracted from apoE-deficient mice and $AgNO_3$ to investigate the earliest phase of AA amyloid deposition. Our present results showing minute amyloid deposits in most mice of both strains at 48 hours after injection and somewhat smaller deposits in the apoE-deficient mice at 72 hours after injection suggest that the existence of apoE might slightly accelerate AA amyloid deposition in its earliest phase. However, the amount of amyloid deposits was not very different between apoE-deficient mice and C57BL/6J control mice after the administration of complete Freund's adjuvant and *M. butyricum*. This discrepancy in the amount of the amyloid deposits between the two induction methods might be caused by a difference in efficacy of induction methods or by accidental unevenness of susceptibility to the stimulation in each mouse. It has been demonstrated that the lipoprotein profiles and composition of lipoproteins are different between normal mice and apoE-deficient mice.[18] Additional studies, including the analysis of serum amyloid A level, lipoprotein profiles, and composition of high-density lipoprotein, are required using various amyloid induction methods to confirm whether apoE accelerates AA amyloid fibril formation from serum amyloid A *in vivo*.

Acknowledgment

We thank Dr. Hara for technical advice on biochemical analysis.

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