Anti-Interleukin 6 Receptor Antibody Inhibits Murine AA-Amyloidosis

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ABSTRACT. ***Objective***. AA-amyloidosis is a severe complication in chronic inflammatory diseases. AA-amyloidosis is caused by the deposition of insoluble fibrils containing AA amyloid protein derived from serum amyloid A (SAA), which is synthesized by inflammatory cytokine stimulation. We examined whether anti-interleukin 6 receptor (IL-6R) antibody prevented the development of AA-amyloidosis in mouse models.

Methods. A transient model was induced by the injection of amyloid enhancing factor (AEF) and adjuvant treatment in C57BL/6 mice. Monoclonal IgG1 antibody, MR16-1, was injected intraperitoneally just once before the injection of AEF and adjuvant. After 2 and 5 weeks, mice were sacrificed and histologically examined. In contrast, a chronic model was induced by AEF injection into IL-6 transgenic mice. One week later, in order to avoid neutralizing antibody production, MR16-1 (200 mg/kg) was injected intravenously. MR16-1 (5 mg/kg) was injected subcutaneously twice a week from the next week. Three and 6 weeks after AEF injection, mice were sacrificed and histologically examined.

Results. In the transient model, amyloid deposition was observed in the spleen, liver, and kidney as early as 2 weeks after treatment. MR16-1 completely prevented amyloid deposition. Although IL-6 production was not suppressed, SAA production was significantly suppressed. In the chronic model, substantial amyloid deposition was seen in multiple organs and tissues as well as the spleen, liver, and kidney. MR16-1 suppressed amyloid deposition in many organs, even when injected one week after AEF injection; it showed a tendency to decrease SAA and IL-6 levels were decreased.

Conclusion. IL-6 is a key cytokine for the induction of AA-amyloidosis, and anti-IL-6R therapy appears promising for the treatment of AA-amyloidosis. (J Rheumatol 2004;31:1132–8)

Key Indexing Terms:
ANTI-INTERLEUKIN 6 RECEPTOR
ANIMAL DISEASE MODELS
AA AMYLOIDOSIS
THERAPY

AA-amyloidosis is a severe complication of chronic inflammatory diseases such as rheumatoid arthritis (RA), Crohn's disease, Castleman's disease, and chronic infections[1-3]. AA-amyloidosis is caused by the deposition of insoluble fibrils containing AA amyloid protein, which is derived from its precursor, serum amyloid A (SAA)[4]. SAA is synthesized predominantly in the liver after stimulation by inflammatory cytokines[5]. SAA circulates as an apolipoprotein to high-density lipoprotein (HDL). When SAA is dissociated from HDL and degraded, it is deposited as AA amyloid fibrils in the extracellular space of vital tissues, leading to organ dysfunction such as renal failure and gastrointestinal tract dysfunction.

In therapy for AA-amyloidosis, treating the basic disease to reduce SAA production is very important. Therefore, antiinflammatory drugs such as nonsteroidals and corticosteroids are commonly used for the prevention of AA-amyloidosis. In addition, dimethyl sulfoxide[6], colchicine[7], and chlorambucil[8] are used for the treatment of human amyloidosis. However, due to their unsatisfactory clinical efficacies and to side effects, more effective and safer new drugs are strongly desired[9,10].

Theoretically, drugs that reduce SAA production would be favorable for the prevention of AA-amyloidosis. Interleukin 6 (IL-6) has been shown to be a key cytokine for SAA production[11]: the blockage of IL-6 caused rapid reduction of serum SAA concentrations in patients with RA[12,13]. We investigated whether anti-IL-6 receptor (IL-6R) antibody, which suppresses IL-6 biological activities, showed a preventive effect on murine AA-amyloidosis models.

MATERIALS AND METHODS

Mice. Male C57BL/6J (7-week-old) mice were purchased from Clea Japan,

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Inc., Tokyo, Japan. Transgenic mice carrying human IL-6 cDNA fused with the H-2L^d promoter were produced and maintained at the Chugai Pharmaceutical Co. Ltd[14]. The animals were specific-pathogen-free and were kept in cages in a room maintained at 24 ± 2°C, and 50–60% relative humidity.

Antibodies. Hybridoma cells that produce rat anti-mouse IL-6R monoclonal IgG1 antibody, MR16-1[15], and hybridoma cells that produce rat anti-dinitrophenyl monoclonal IgG1 antibody, KH-5, were established in our laboratories. IgG fraction was purified from ascites fluid of a pristane-treated BALB/c nude mouse using a protein A column.

Preparation of amyloid enhancing factor. Amyloid enhancing factor (AEF) was prepared from amyloidotic mouse livers and spleens according to the method of Hoshii, *et al*[16]. Briefly, male C57BL/6 mice were injected with 0.2 ml of complete Freund's adjuvant (CFA) containing 3.13 mg of *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI, USA) and 0.5 ml of AEF (kindly provided by Dr. H. Kawano, Yamaguchi University). Two weeks later, mice were sacrificed and livers and spleens were homogenized with 8 ml of 4 mol/l glycerol, 0.01 mol/l Tris/HCl (pH 7.6) per gram organ. The homogenates were shaken for 1 h at 4°C and then centrifuged at 20,000 g for 1 h at 4°C. The supernatant was dialyzed overnight against phosphate buffered saline in a Spectra/Pro 3 Molecularporous dialysis membrane (Spectrum, Houston, TX, USA) with a molecular weight cutoff of 3500.

Transient amyloidosis model. Male, 8-week-old C57BL/6 mice were injected subcutaneously with 0.2 ml of CFA containing 3.13 mg of *M. butyricum* (Difco), following intraperitoneal injection of AEF (0.5 ml). After 2, 5, and 10 weeks, mice were sacrificed, and organs (spleen, liver, and kidney) were fixed with 20% neutral formaldehyde.

In the antibody treatment study, MR16-1 was injected intraperitoneally just before injection of AEF and adjuvant. Control mice received saline or isotype-matched rat IgG1 (KH-5). After 2 and 5 weeks, mice were sacrificed and organs were fixed with 20% neutral formaldehyde.

Chronic amyloidosis model. Female, 8-week-old IL-6 transgenic mice were injected intraperitoneally with 0.5 ml of AEF. After 1, 3, and 6 weeks, mice were sacrificed and organs (spleen, liver, kidney, digestive organs, adrenal gland, and thyroid gland) were fixed with 20% neutral formaldehyde.

In the antibody treatment study, mice were injected intraperitoneally with 0.5 ml of AEF, and one week later, mice were divided into 2 groups (KH-5 and MR16-1 groups). Since KH-5 and MR16-1 are heterogenous proteins to mouse, the administration cannot be continued for a long time because of neutralizing antibody production. In order to avoid antibody production, either MR16-1 or KH-5 (200 mg/kg) was injected intravenously and tolerance was induced. After that, the same antibody (5 mg/kg) was injected subcutaneously twice a week from the next week. This procedure suppressed antibody production[17]. Three and 6 weeks after AEF injection, mice were sacrificed and organs were fixed with 20% neutral formaldehyde.

IL-6 and SAA concentrations in serum. IL-6 and SAA concentrations in serum were measured by ELISA kit (mouse and human IL-6; Amersham, SAA; BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions.

Histological examination. After fixing organs with 20% neutral formaldehyde, organs were embedded in paraffin, sectioned, and stained with alkaline Congo red. Histological grading under polarized light was carried out by the blinded investigator, using a grading system of 0–4 (0: no detectable deposition, 1: slight amyloid deposition, 2: weak deposition, 3: moderate deposition, 4: substantial deposition).

Statistical analysis. The statistical significance of the differences was analyzed by unpaired t test and Wilcoxon 2-sample test using a software package (Statistical Analysis System; SAS Institute Japan Ltd., Tokyo, Japan). Significance was set at a p level of less than 0.05.

RESULTS

Amyloid deposition in the transient model. Amyloid deposition was observed from 2 weeks after the injection of AEF and adjuvant and it remained at 10 weeks, although the amount decreased. The apparent amyloid deposition was observed in the spleen, liver, and kidney, but it was never seen in the gastrointestinal organs. In addition, neither treatment with AEF nor with adjuvant alone induced any amyloid deposition.

Previously, we observed that peak IL-6 production was induced after 7 days of adjuvant injection into mice[18]. In this experiment, peak IL-6 production was observed at 7 days after the injection of AEF and adjuvant. However, there was another peak at 5 h after injection of AEF and adjuvant (Figure 1B). The SAA level gradually increased, reached a peak at 1 week after the treatment, then decreased (Figure 1A). Neither tumor necrosis factor-α (TNF-α) nor IL-1ß was detectable.

Effect of MR16-1 on transient amyloidosis model. MR16-1 was injected before the injection of AEF and adjuvant. Control mice received saline or KH-5. After 2 and 5 weeks, mice were sacrificed and amyloid deposition was histologically examined. In the control mice, a marked amyloid deposition was observed in the spleen, liver, and kidney. However, no amyloid deposition whatsoever was observed in the MR16-1-treated mice (Figure 2).

Serum concentrations of SAA and IL-6 were measured at 5 h and 1 week after injection of AEF and adjuvant. Serum IL-6 concentrations were clearly augmented by MR16-1. However, the production of SAA was significantly suppressed by MR16-1 treatment (Figure 3).

Induction of amyloidosis in IL-6 transgenic mice. Female, 8-week-old IL-6 transgenic mice received 0.5 ml of AEF intraperitoneally. Mice were sacrificed 1, 3, and 6 weeks after AEF injection, and the amyloid deposition was examined. Amyloid deposition was not observed in IL-6 transgenic mice without AEF treatment. In AEF-injected IL-6 transgenic mice, the degree of amyloid deposition became stronger depending on the lapse of time after AEF injection. Amyloid deposition was observed not only in the liver, spleen, and kidney but also in the digestive organs, adrenal gland, and thyroid gland (Figure 4).

An age-related increase in human IL-6 and SAA was observed (Figure 5). Two mice in the AEF-injected group died between 3 weeks and 6 weeks after AEF injection.

Effect of MR16-1 on chronic amyloidosis. MR16-1 treatment showed a tendency to suppress the amyloid deposition at both 3 and 6 weeks after AEF injection in all organs examined compared with KH-5 treatment, although findings in some organs were not statistically significant (Figure 4).

SAA concentrations in the MR16-1-treated group were lower than those in the KH-5-treated group, although the differences were not statistically significant. And the MR16-1 treatment reduced serum human IL-6 concentrations compared with the KH-5 treatment (Figure 6).

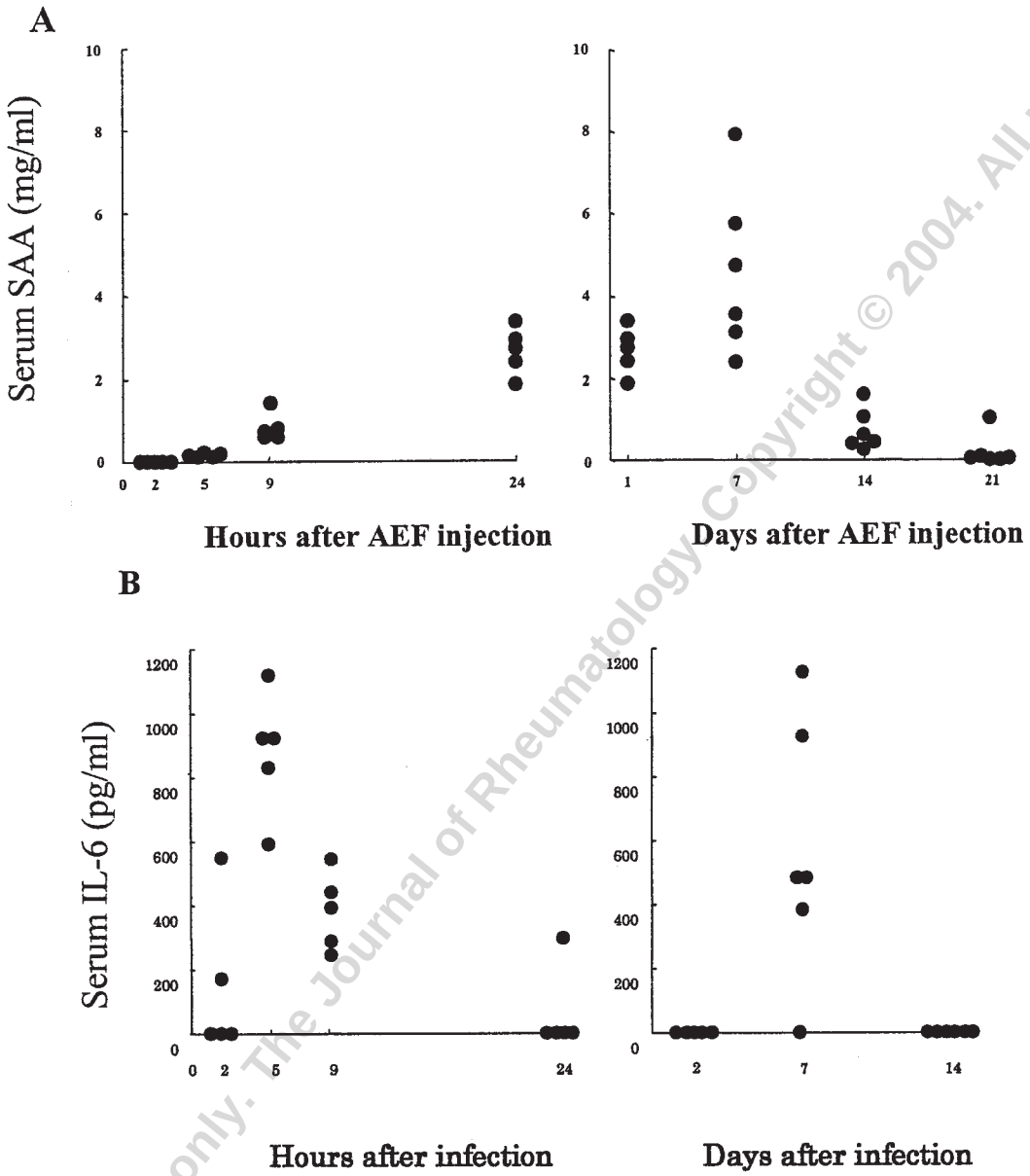

Figure 1. Serum SAA (A) and IL-6 (B) concentrations in transient AA-amyloidosis model. C57BL/6 mice were treated with amyloid enhancing factor (AEF) and adjuvant. Serum was collected at 2, 5, 9, and 24 h and 2, 7, 14, and 21 days after treatment. Serum concentration of mouse IL-6 and SAA concentrations were measured by ELISA. Each group consisted of 5 mice, except for 14 and 21 days (6 mice).

DISCUSSION

We examined the inhibitory effect of anti-IL-6R antibody on 2 mouse models of AA-amyloidosis, i.e., AEF + adjuvant-injected mice and AEF-injected IL-6 transgenic mice. Histological examination clearly showed that MR16-1 suppressed amyloid deposition in multiple organs such as the spleen, liver, kidney, and the gastrointestinal organs in both models (Figures 2 and 4). These beneficial effects of MR16-1 are due to the reduction of SAA production.

The AEF + adjuvant-induced AA-amyloidosis model is widely used for the analysis of pathology and experimental therapy. Our data showed that the amyloid deposition was observed only in spleen, liver, and kidney, and that the amyloid deposition and SAA production were transient. Adjuvant can induce several kinds of cytokine, such as IL-6, IL-1, TNF-α, and so on. However, we showed that MR16-1 completely suppressed amyloid deposition and SAA production, indicating that IL-6 is an essential cytokine for the onset of AA-amyloidosis. Although there was a biphasic elevation of IL-6 (at 5 h and Day 7), SAA produc-

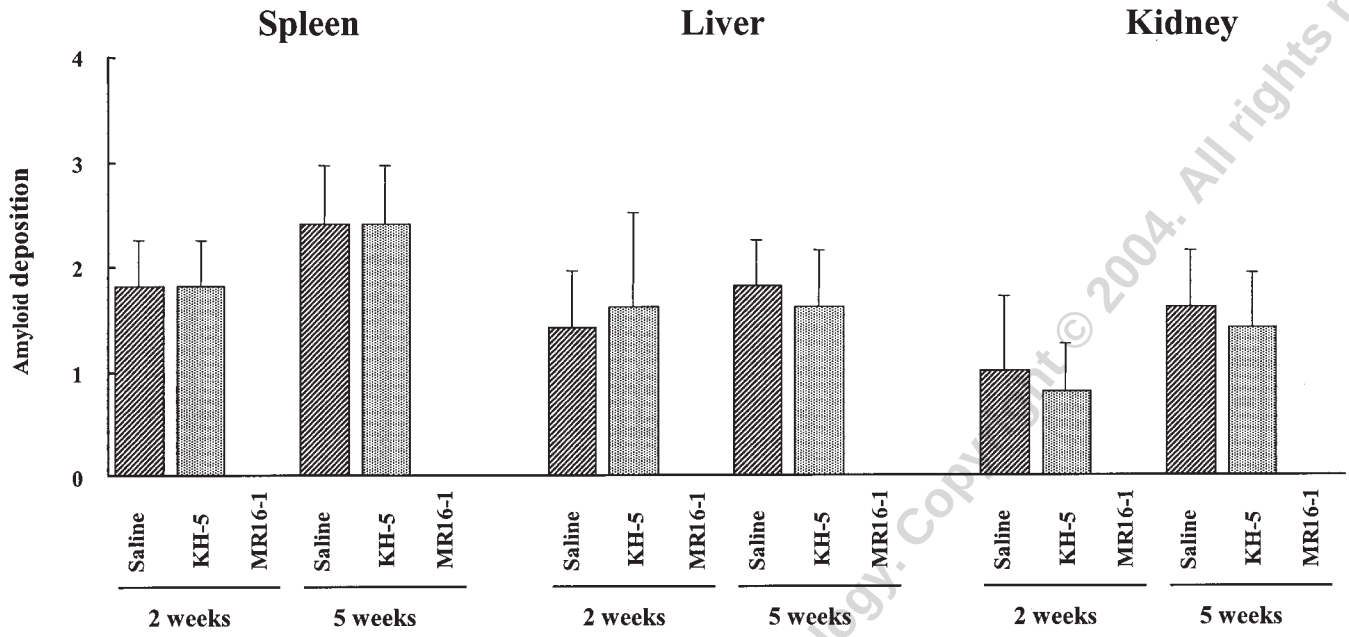

Figure 2. Effect of MR16-1 on amyloid deposition. Saline, KH-5, and MR16-1 were injected IP before the injection of AEF and adjuvant treatment. Mice were sacrificed at 2 and 5 weeks after treatment, followed by histological examination using a grading system of 0–4 (0: no detectable deposition; 1: slight amyloid deposition; 2: weak deposition; 3: moderate deposition; 4: substantial deposition). The columns and vertical lines indicate mean and SD.

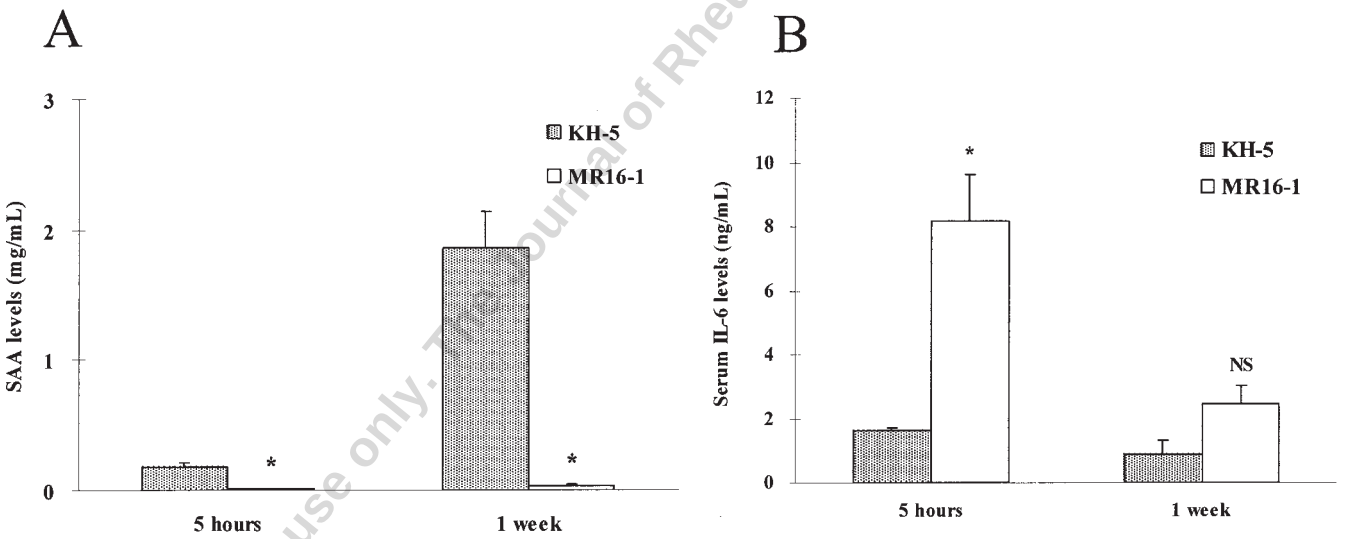

Figure 3. Effect of MR16-1 on serum SAA (A) and IL-6 (B) concentrations in the transient AA-amyloidosis model. C57BL/6 mice were treated with AEF and adjuvant. MR16-1 or KH-5 were injected IP before injection of AEF and adjuvant. Mice were sacrificed at 5 h and 1 week after treatment, and then serum IL-6 and SAA concentrations were measured by ELISA. Columns and vertical lines indicate the mean and SE of 5 mice.

tion reached a maximum on Day 7. The reason for this phenomenon is not clear.

In contrast to the transient model, the model using IL-6 transgenic mice showed that the degree of amyloid deposition was extremely strong, and deposition was seen in multiple organs (Figure 4). Further, since IL-6 was produced constantly, SAA production also continued for a long time. Therefore, this model is more comparable to human AA-amyloidosis than the transient model.

MR16-1 also showed a suppressive effect on amyloid deposition and SAA production in this model, although the degree of suppression was weaker than in the transient model. This may be due to the following: (1) IL-6 transgenic mice already had large amounts of IL-6 at the injection of MR16-1, whereas IL-6 was not detectable in the transient model. (2) In the chronic model, amyloid deposition was already present at the initiation, whereas in the transient model, the experiment was started under the condition of no amyloid deposition.

Solomon, *et al* reported that transgenic mice carrying

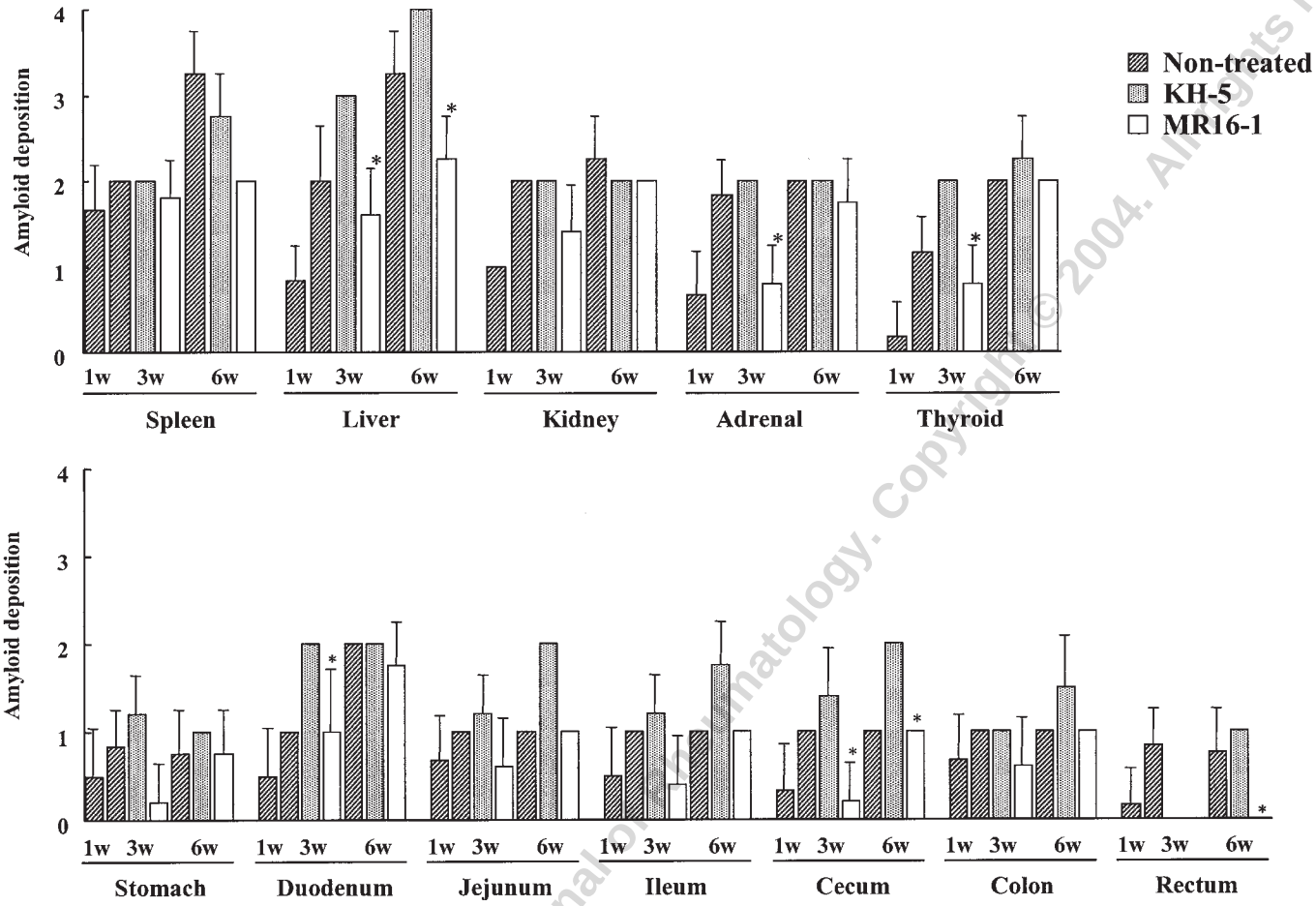

Figure 4. Amyloid deposition in AEF-injected IL-6 transgenic mice. Two separate experiments are combined in this figure. Non-treatment: AEF was injected IP into IL-6 transgenic mice. Mice did not receive antibody treatment. Mice were sacrificed at 1, 3, and 6 weeks after AEF injection, followed by histological examination. Antibody treatment: One week after AEF injection, mice were divided into 2 groups. MR16-1 and KH-5 (200 mg/kg) were injected intravenously. After that, 5 mg/kg antibody was injected subcutaneously twice a week from the next week. Three and 6 weeks after AEF injection, mice were sacrificed for histological examination. Statistical significances between KH-5 and MR16-1 were analyzed by Wilcoxon 2-sample test. *$p < 0.05$. Columns and vertical lines indicate mean and SD.

human IL-6 gene under the control of MT-I naturally developed amyloidosis in the spleen, liver, and kidney by 3 months of age, but not in IL-6 transgenic mice under the control of Eμ enhancer[19]. Our transgenic mouse carrying human IL-6 gene under the control of the H-2L^d promoter did not induce naturally-occurring amyloid deposition. Although the precise reason for this discrepancy is unknown, the difference in the site of IL-6 expression may be a factor. Further, the differences in mouse strain are important. The background strains of MT-I/IL-6, Eμ/IL-6, and H-2L^d/IL-6 transgenic mouse are (C57BL/6 x DBA2)F1, BALB/c, and C57BL/6J, respectively. Indeed, the strain difference in the induction of amyloid deposition has been reported[20].

AEF in its crude form is a homogenate of amyloidotic or pre-amyloidotic tissues[21,22]. Neither the identification of the active component of AEF nor the mechanism of action has been conclusively determined, despite a considerable history of experimentation[23,24]. As severe amyloidosis can be induced by only one injection of AEF and never occurs spontaneously in our IL-6 transgenic mice, our mice are a suitable tool for the analysis and identification of AEF.

MR16-1 treatment increased serum concentrations of IL-6 in the transient model; however, it almost completely suppressed the SAA production. An increase in IL-6 concentrations by anti-IL-6R antibody was reported in other cases[18]. This phenomenon may be due to blockage of IL-6 clearance from the bloodstream rather than augmentation of IL-6 production. In contrast, MR16-1 decreased serum IL-6 concentrations in the chronic model. In IL-6 transgenic mice, age-related enlargement of lymphoid organs is observed, and this enlargement is synchronized with serum IL-6 levels; moreover, continuous injection of MR16-1 suppressed lymphadenopathy and IL-6 production, suggesting that MR16-1 suppressed IL-6 production via the suppression of an increase of IL-6-producing cells[17].

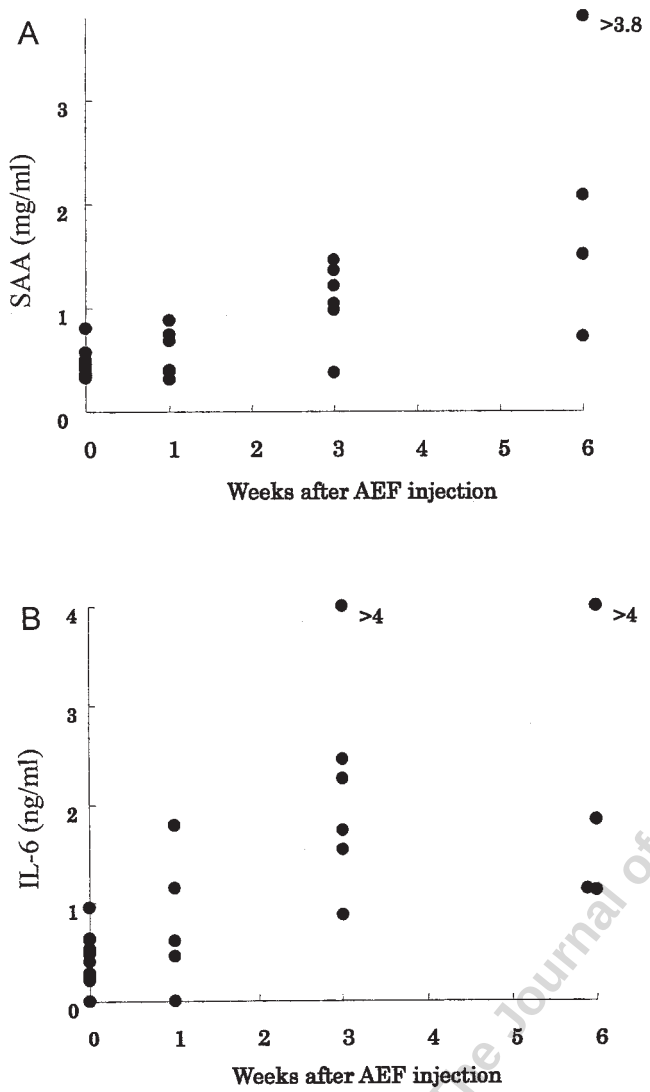

Figure 5. Serum SAA (A) and IL-6 (B) concentrations in the chronic AA-amyloidosis model. AEF was injected IP into IL-6 transgenic mice. Mice were sacrificed at 1, 3, and 6 weeks after AEF injection, and serum human IL-6 concentrations were measured by ELISA.

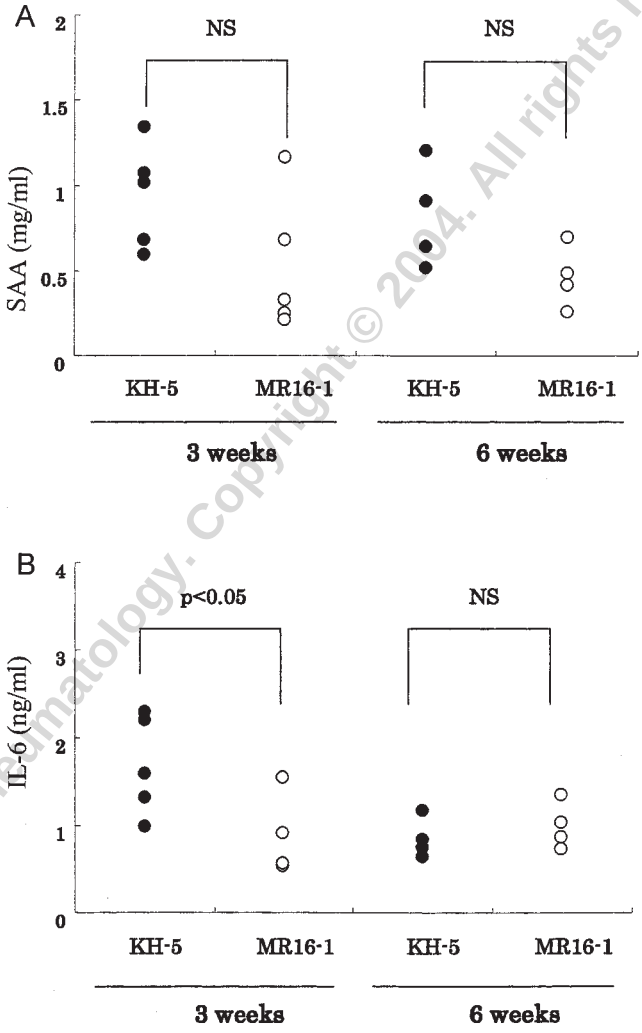

Figure 6. Effect of MR16-1 on serum SAA (A) and IL-6 (B) concentrations in the chronic AA-amyloidosis model. AEF was injected IP into IL-6 transgenic mice. One week later, mice were divided into 2 groups. MR16-1 or KH-5 (200 mg/kg) was injected intravenously. After that, MR16-1 or KH-5 antibody (5 mg/kg) was injected subcutaneously twice a week from the next week. Three and 6 weeks after AEF injection, mice were sacrificed and serum human IL-6 concentrations were measured by ELISA. Statistical significance was analyzed by unpaired t test. NS: not significant.

In Japan, AA-amyloidosis is observed most frequently in patients with RA. Since AA-amyloidosis induces multiorgan dysfunction such as nephritic syndrome, heart failure, and severe diarrhea, prognosis is poor. Humanized anti-IL-6R antibody, MRA, is very useful for treatment of patients whose active disease is resistant to conventional therapy[25]. MRA not only ameliorated morning stiffness and swollen joint score, but also decreased serum SAA levels in those patients, indicating that MRA may have anti-amyloidosis activity.

As amyloid deposition is reversible, it is possible to cure amyloidosis if SAA production is prevented[26,27]. Since anti-IL-6R antibody completely suppressed the production of SAA, anti-IL-6R antibody therapy may be an attractive treatment for secondary amyloidosis.

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