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Effect of selective IL-6 inhibitor Am-80 on experimental autoimmune encephalomyelitis in DA rats

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KEY WORDS allergic encephalomyelitis; Am-80; interleukin-6; interleukin-10; transforming growth factor beta; tumor necrosis factor; nitric-oxide synthase

ABSTRACT

AIM: To observe the role of interleukin (IL)-6 in the development of experimental autoimmune encephalomyelitis(EAE). **METHODS**: DA rats were immunized by injecting bovine myelin basic protein(MBP). mRNA of cytokines, such as IL-6, IL-10, TNF-α, TGF-β1, IFN-γ, and iNOS, were detected by RT-PCR. MBP was injected into ear to induce delayed type cutaneous hypersensitivity response (DTH). Histological studies were performed on the spinal cord with HE staining. Nitric oxide (NO) production from cultured murine macrophage clones was stimulated with LPS plus IFN-γ. **RESULTS**: DA rats developed EAE disease with a peak of severity on d 13 and d 14. Am-80 (1.0, 3.0 mg/kg), a selective IL-6 inhibitor, inhibited the symptoms in terms of deterioration as observed by the clinical score, body weight and histological findings, in a dose-related manner. A high dose of Am-80 (3.0 mg/kg for 12 d) did not completely inhibit the disease, but delayed the symptoms and enhanced the delayed response. By prolonging the duration of treatment (18 d), Am-80 inhibited the onset of EAE during administration, but the symptoms of EAE appeared after the administration was stopped. Am-80 administered for 12 d inhibited the DTH response on d 11 but not on d 22. RT-PCR studies demonstrated a strong expression of IFN-γ, IL-6, IL-10, TGF-β1, TNF-α, and iNOS mRNA in spinal cord 13 d after immunization. However IFN-γ, IL-10, TNF-α, and iNOS mRNA expression (on d 13) was suppressed by Am-80, except in the case of IL-6, hence the effect of Am-80 on the expression of IL-6 mRNA was examined in additional experiments. After Am-80 was administered for 12 d or 18 d, the expression of IL-6 mRNA was inhibited on d 12 or d 18, but increased on d 13 or d 19, respectively. **CONCLUSION**: These findings suggest that inhibition of EAE by Am-80 is initiated by inhibition of IL-6 production.

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INTRODUCTION

Am-80, 4-[(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthyl) carbamoyl] benzoic acid, is a novel synthetic retinoic acid with potent bioactivity and stability[1]. It is approximately ten times more potent than all trans-retinoic acid in differentiating acute promyelocytic leukemia cells[2]. In our previous study, Am-80 strongly inhibited the development of collagen-induced arthritis through the selective inhibition of IL-6 production[3].

Experimental autoimmune encephalomyelitis (EAE), a model for T-cell-mediated autoimmune disease shares in many features with the human disease, multiple sclerosis (MS), and is characterized by CNS inflammation and demyelination of neurons especially in the spinal cord. The detailed mechanism of development of EAE is still unclear. Cytokines and Nitic oxide (NO) play key roles during the onset and recovery process of this disease[4,5]. Pro-inflammatory cytokines, IFN-γ and TNF-α, play critical roles in the pathogenesis of EAE[6-8], while immunoregulatory cytokines, such as TGF-β and IL-10, are related to the recovery process from this disease[9-12]. High levels of NO production and iNOS mRNA expression have been correlated with pathogenesis of this disease[13-17].

Here, we attempted to investigate the effect of the

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selective IL-6 inhibitor, Am-80, on EAE disease in DA rats and to analyze the mechanism involved in the onset of the symptoms, especially focusing on alterations in cytokines and iNOS mRNA expression levels during the development of this disease.

MATERIALS AND METHODS

Animals Female DA rats (7 – 8 wk old) obtained from Japan SLC Inc (Hamamatsu, Japan), were housed in an air-conditioned room at (22 ± 1) ℃ and fed a standard laboratory diet and given water *ad lib*. All experiments were carried out following the guidelines for the care and use of experimental animals by the Japanese Association for Laboratory Animal Science in 1987.

Reagents Am-80 was synthesized by Prof SHUDO Koichi (University of Tokyo). Am-80, white powder, was suspended in 0.5 % carboxylmethyl cellulose solution for *in vivo* studies or dissolved at different concentrations in dimethyl sulfoxide for *in vitro* studies. Bovine myelin basic protein (MBP, Sigma, St Louis, MO, USA), complete Freund's adjuvant (Nacalai Tesque Inc, Kyoto, Japan), prednisolone acetate (Pred, Shionogi Osaka, Japan), aminoguanidine hemisulfate (AG, RBI, Natick, MA, USA), lipopolysaccharide (LPS, Difco, Detroit, MI, USA), *Mycobacterium tuberculosis* H37Ra (*M tuberculosis*, Difco), interferon-gamma (IFN-γ, recombinant murine, Life Technologies, Gaithersburg, MD, USA), Griess Reagent System (Promega Co, Madison, WI, USA), ISOGEN (Wako Co Ltd, Osaka, Japan), SuperScript™ RNase H reverse transcriptase (Life Technologies) and TaKaRa Taq™ (Takara Shuzo Co Ltd, Shiga, Japan) were purchased commercially.

Induction of EAE EAE was induced into the DA rats according to the method previously described[18]. Briefly, MBP was dissolved in phosphate buffered saline, and mixed with an equal volume of complete Freund's adjuvant. Then *M tuberculosis* was added to the mixture and emulsified. Emulsion containing 100 μg MBP and 200 μg *M tuberculosis* was injected subcutaneously in both hind foot pads of the animals. To evaluate the efficacy of Am-80, the drug was administered orally every day for indicated periods after immunization with MBP.

Body weights were measured and clinical symptoms were scored as follows: 0, normal; 1, flaccid tail; 2, partial paralysis of hind feet; 3, complete paralysis of hind feet; 4, paralysis of fore feet.

Delayed type cutaneous hypersensitivity response (DTH) The DTH response to MBP was measured with the presence (d 11) and absence (d 22) of the treatment of Am-80 after immunization. The spontaneous response to MBP was also examined in non-immunized rats. MBP solution 0.4 g/L, 50 μL (containing 0.2 mol/L NaCl and 0.05 mol/L Tris-HCl, pH 7.5), was injected intradermally into the skin of the left ear and an equal volume of the buffer solution was injected into the skin of the right ear. After 48 h, the thickness of both ears was measured with an Upright Dial Thickness Gauge (Ozaki Seisakusyo, Tokyo, Japan).

Histological studies Histological studies were performed on the spinal cord of rats from each group. Under anesthesia, the rats were perfused through the ascending aorta with 0.9 % saline followed by 5 % formalin and the spinal cord were isolated and post-fixed in 10 % formalin. Ten μm sections stained with hematoxylin and eosin were then assessed by light microscopy.

Analysis of cytokine mRNA expression in spinal cord by RT-PCR Changes of cytokine mRNA levels in spinal cord were assessed by RT-PCR using a thermal cycler (Bio Metra Trio-Thermoblock, Bio Metra Co, Ltd, Gottingen, Germany). Rats were killed and the spinal cords were rapidly removed for RNA extraction. Using ISOGEN, total RNA was prepared from the nerve tissue of the rats. The amount of total RNA in each sample was measured spectrophotometrically at a wavelength of 260 nm and the quality of RNA was checked by electrophoresis. RT-PCR was performed as follows. Total RNA was reverse-transcribed with Random hexamers, 5-fold first strand buffer plus dNTP, dithiothreitol and SuperScript™ RNase H-reverse transcriptase. Each cDNA sample was amplified in a total volume of 50 μL containing 1 μmol/L of each primer with TaKaRa Taq™. The mixture was overlaid with mineral oil and subjected to 35 or 40 PCR cycles in a thermal cycler. RT-PCR was performed on glyceraldehyde 3-phosphate dehydrogenase (G3PDH), IFN-γ, IL-6, IL-10, TGF-β1, TNF-α, and iNOS. The sequences of the primers are listed in Tab 1. The PCR products were resolved by electrophoresis and stained with ethidium bromide. Samples were obtained from 3 – 4 rats. RT-PCR was semi-quantified by densitometrically scanning photo negatives produced using a Polaroid camera (Poraloid 665 film, Polaroid Corp, Cambridge, MA, USA). For relative semi-quantitation, the densitometric value of each cytokine was normalized to that of the house-keeping gene, G3PDH, and then levels were compared between groups.

Tab 1. Sequence of primers used in this experiment and the number of PCR cycles. (Upper is sense and lower is antisense, from 5′ to 3′)

Primer	Sequence (5′ – 3′)	Length (bp)	PCR cycle
G3PDH	TGT ATC CGT TGT GGA TCT GAC ATG C CCC TGT TGC TGT AGC CAT ATT CAT TGT C	254	35
IFN-γ	ATG AGT GCT ACA CGC CGC GTC TTG G GAG TTC ATT GAC AGC TTT GTG CTG G	405	35
iNOS	TGA TGT GCT GCC TCT GGT CT ACT TCC TCC AGG ATG TTG TA	279	35
IL-6	GAT GTT GTT GAC AGC CAC TGC GAG TTG GAT GGT CTT GGT CCT	526	40
IL-10	TTA CCT GGG TTC TCC AAG CCT T CTT CAC AGA GAA GCT CAG T	201	35
TGF-β1	ACT ACT GCT TCA GCT CC CAG GAG CGC ACG ATC AT	313	35
TNF-α	GGA AAG CAT CAT CCG AGA TG AAA GTA GAC CTG CCC GGA CT	682	35

In addition, a linear correlation between RNA input and PCR product was examined. Fair linearity was obtained between the density value of PCR product and RNA input. All PCR amplifications were performed at least twice with multiple sets of experimental RNA.

NO production from cultured murine macrophage clones stimulated with LPS plus IFN-γ The murine macrophage like cell line, RAW264.7, was maintained at 37 ℃ in an atmosphere of 5 % CO_2 and cultured in RPMI-1640 (Gibco) with 10 % fetal calf serum, 2 mmol/L *L*-glutamine, 100 kU/L penicillin G and streptomycin sulfate 100 mg/L. The cells were seeded at 4×10^8 cells/L onto 6-well-plate in 5 mL of fresh culture medium and then incubated at 37 ℃ overnight. The medium was replaced with 5 mL of fresh medium containing 100 mg/L LPS with 10 kU/L IFN-γ. The cells were further incubated at 37 ℃ for 48 h. Then the culture supernatant was collected and stored at 4 ℃ until use. NO levels were determined by nitrite assays. Nitrite was measured according to the method of Griess Reagent System. Nitrite standards were diluted in media and were unaffected by the concentrations of drugs used in these studies.

Statistics Results were expressed as $\bar{x} \pm s$. Data were evaluated by either Student's t test or Dunnet's test after examining the variances using the F test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Am-80 on the EAE disease Clinical score and body weight of control group animals deteriorated from d 10 after immunization and peaked on d 13 – 14 (Fig 1A, termed as the first response). At this time, Am-80 (1.0, 3.0 mg/kg) administered for 12 d inhibited the severity of the disease in a dose-dependent manner. Histological examination revealed that Am-80 inhibited the infiltration of inflammatory cells into the spinal cord during treatment (Fig 2).

The severity of EAE, indicated as a clinical score, however, increased after stopping the administration of Am-80 (3.0 mg/kg) and reached the peak on d 17 – 19 (Fig 1A). Delayed response in the Am-80 (3.0 mg/kg, for 12 d) treated group was termed as the second response. Treatment with Pred 5.0 mg/kg for 12 d significantly inhibited clinical score elevation without a delayed response. Prolonged treatment with Am-80 for 18 d gave similar results (Fig 1B). The animals, treated with Am-80 3.0 mg/kg for 18 d after immunization did not exhibit any changes in clinical score and body weight during the treatment period, but after stopping the administration, the clinical score elevated. Compared with the control group, the severity of the disease in the Am-80 treated rats did not aggravate after treatment.

Effect of Am-80 on DTH response Am-80 (0.3, 1.0, and 3.0 mg/kg) administered for 12 d inhibited DTH response in a dose-dependent manner on d 11 but not on d 22 (Fig 3).

Effects of Am-80 on the expression of cytokines and iNOS mRNA in spinal cord mRNA levels of IFN-γ, IL-6, IL-10, TGF-β1, TNF-α and iNOS in spinal cords from the control group correlated with the disease symptoms. High levels of mRNA expression of cytokines and iNOS were detected both in the

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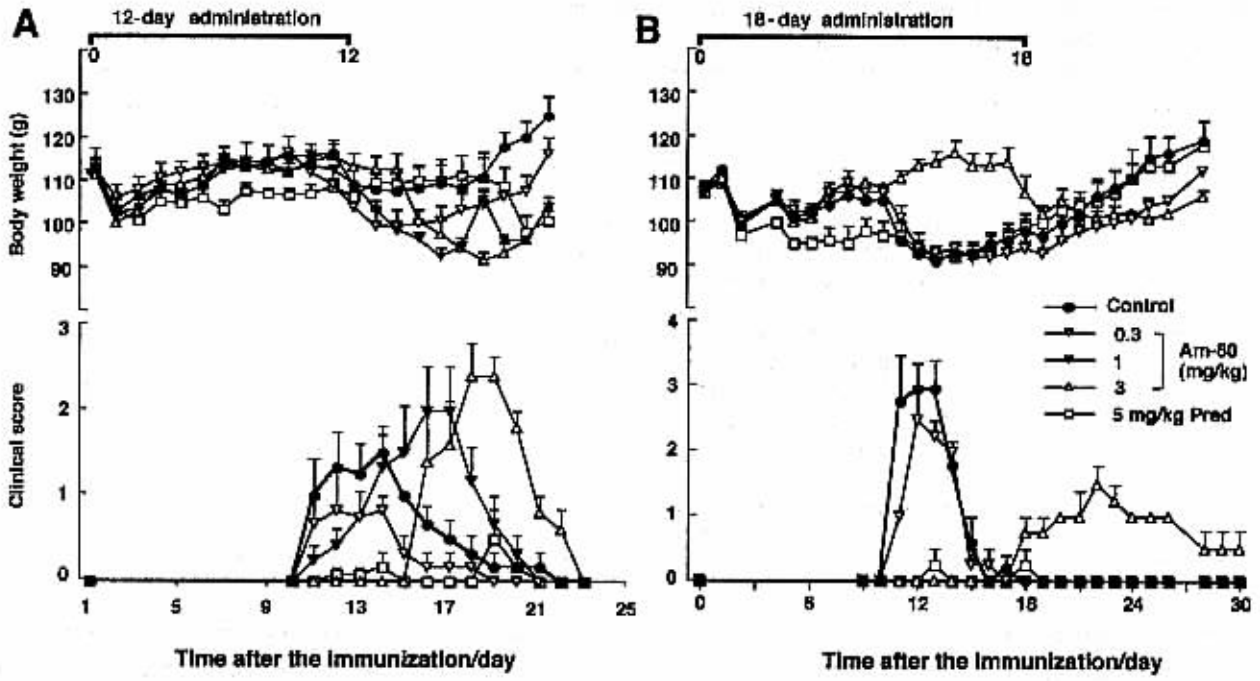

Fig 1. Effects of Am-80 and prednisolone (Pred) on EAE in DA rats as measured by body weight and clinical score. Am-80 and Pred were administered *po* daily for 12 d (A) or 18 d (B). $n=6$. $\bar{x} \pm s$.

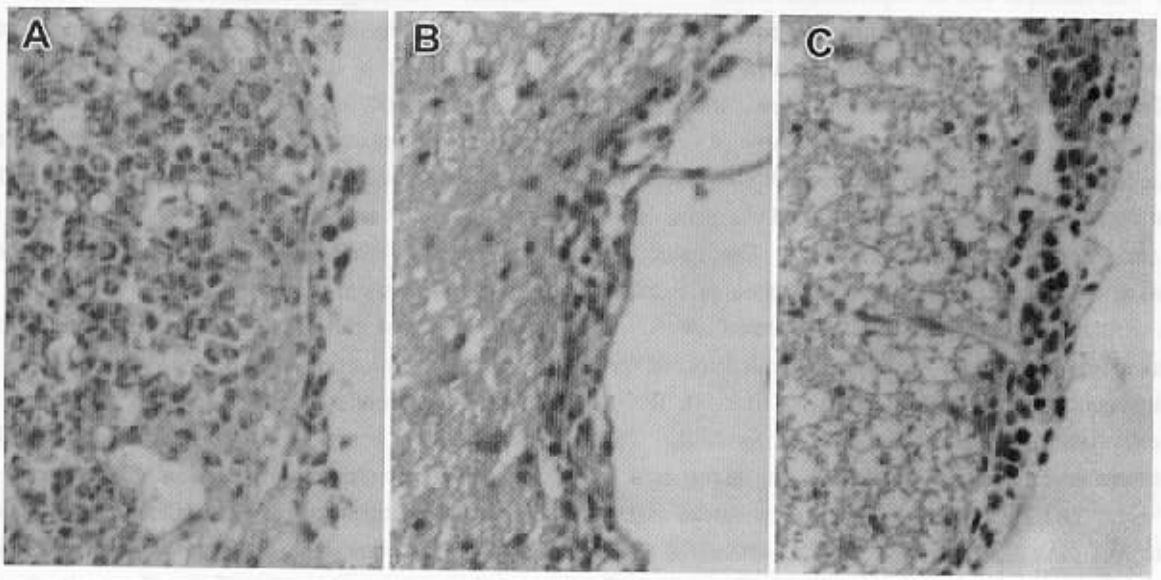

Fig 2. Monocytic infiltration in the spinal cord section from rats in the control group (A), Am-80 (3.0 mg/kg) treated group (B), and Pred (5.0 mg/kg) treated group (C). ×100.

first and the second response in the spinal cords. Pred inhibited mRNA expression of cytokines and iNOS. This data suggested a positive correlation between mRNA expression levels in nerve tissues of cytokines and iNOS, and disease severity (Fig 4, 5).

Am-80 inhibited the mRNA expression of cytokines and iNOS except for IL-6 in the spinal cord in the first response, while in the second response, the mRNA expression levels of all cytokines and iNOS in the Am-80 treated group increased. However, the effect of Am-80 on IL-6 mRNA level was different from other cytokines. Experiments were then conducted to compare the effect of Am-80 on IL-6 mRNA expression during the administration period and after the administration of drug. Results were shown in Fig 6. Am-80 (3.0 mg/kg) inhibited IL-6 mRNA expression during the administration period (d 12

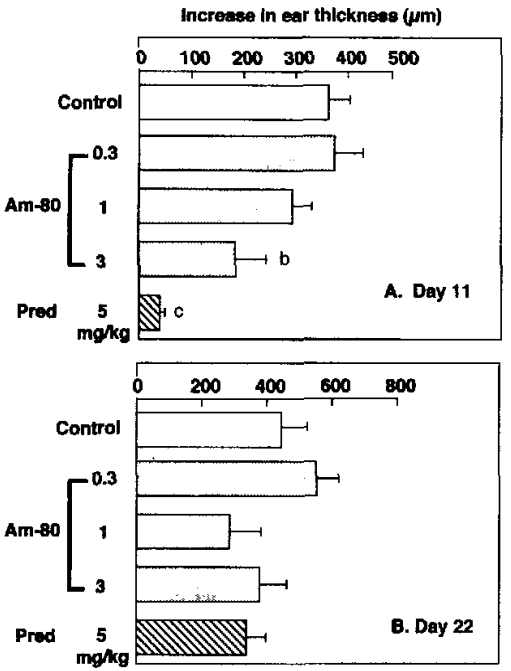

Fig 3. Effects of Am-80 and Pred on MBP-induced DTH response in DA rats (A, d 11; B, d 22). $n=5-6$ rats. $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control (Dunnet).

and 18). One day after the treatment with the drug (d 13 and d 19) was stopped, the levels of IL-6 mRNA increased.

Effects of Am-80 on iNOS mRNA expression and nitrite production To determine the effect of Am-80 on the production of NO during the development of EAE, we examined iNOS mRNA expression levels *in vivo* and NO production *in vitro*.

iNOS mRNA expression levels in spinal cord positively correlated with the disease severity. In the first response, Am-80 inhibited iNOS mRNA expression in the spinal cord. While during the second response, after cessation of Am-80 treatment, iNOS mRNA expression levels increased. LPS plus IFN-γ stimulated iNOS mRNA expression in cultured RAW264.7 cells and increased nitrite production in the culture medium. Only high dosage (100 mmol/L) of Am-80 significantly inhibited nitrite production (Fig 8).

DISCUSSION

Our findings indicate that Am-80 inhibition of EAE severity correlates with inhibition of the transient expression levels of cytokines and iNOS in the spinal cord and strongly suggests that IL-6 may play an initial role during the onset of EAE.

Increased levels of IL-6 in nerve tissue have been linked to pronounced reactive gliosis[19] and parenchymal CNS injury[20]. IL-6 can be induced by IL-1 and IFN-γ[21], inhibited by IL-10[22], and regulated by expression of other cytokines, such as TNF-α[23], suggesting that IL-6 has a dual regulatory effect. Kennedy *et al* (1992)[11] reported that the level of IL-6 peaked early during the acute phase of the disease and started to wane before the peak of the disease, while IL-10 mRNA levels were still elevated during the recovery period in mice. They concluded that IL-10 mRNA expression correlates with recovery but they did not pay much attention to the role of IL-6. Changes in cytokine levels with the development of EAE have been previously reported[24,25,15]. In the present experiment, we obtained similar results about various kinetic expressions of IFN-γ, IL-10, and TNF-α as well as data suggesting a role for IL-6 during the initiation of EAE, by using the selective IL-6 inhibitor Am-80. But we failed to find a previously demonstrated relationship between TGF-β1 mRNA and EAE in mice[4,9,12]. Recently, Diab *et al* (1997)[26] have reported that difference of cytokine levels in two strains of rats, Lewis and DA, during EAE. TGF-β1 does not play an important role for the onset of EAE in DA rats. In this experiment, we demonstrated that a high dosage of Am-80 (3.0 mg/kg for 12 d) does not inhibit, but just delays the onset of EAE and enhances the severity of the delayed symptoms. Prolonged administration of Am-80 for 18 d also did not completely inhibit the development of disease (Fig 1). Meanwhile, Am-80, as a selective IL-6 inhibitor, did not inhibit IL-6 mRNA expression one day after cessation of the administration (on d 13) and this unique high level of mRNA IL-6 was followed by the onset of EAE. This suggests that there is a rapid elevation of IL-6 mRNA expression after stopping inhibition of IL-6 transcription during the development of the EAE, and this results in a delayed and enhanced second response. Further studies are needed to confirm this phenomenon.

We demonstrate that IL-6 may be an initial factor during the onset of EAE. The early expression of IL-6 in the acute phase of EAE was also reported by Kennedy *et al* (1992). Here we examined in detail the trigger role of IL-6 during the development of EAE. In this case, EAE onset followed the expression of IL-6 mRNA

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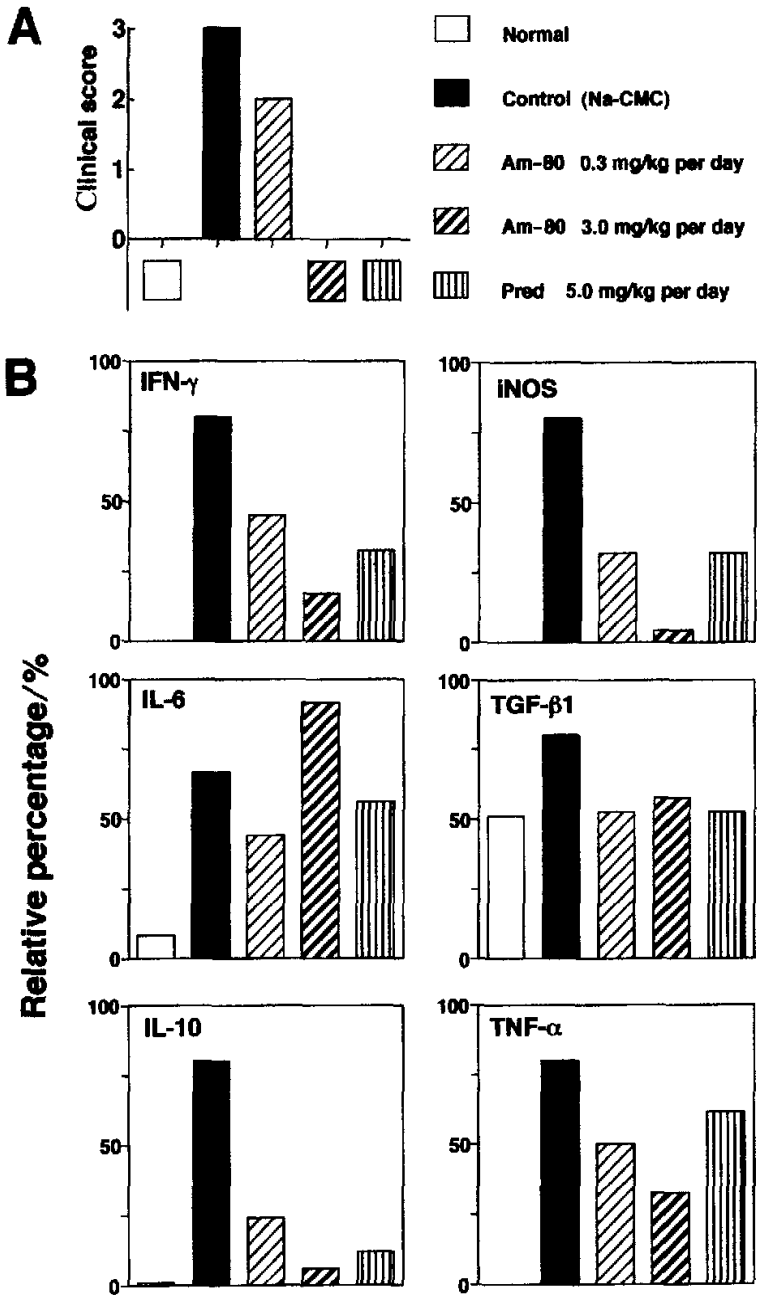

Fig 4. Effects of Am-80 and Pred on the mRNA expression of cytokines and iNOS in the spinal cord of DA rats 13 d after immunization. Am-80 and Pred were administered *po* daily for 12 d. A. Clinical score of each group tested. B. Changes in mRNA levels of different cytokines and iNOS are represented as the relative percentage of levels compared with that of G3PDH. Each value represents the mean obtained from 3 - 4 samples.

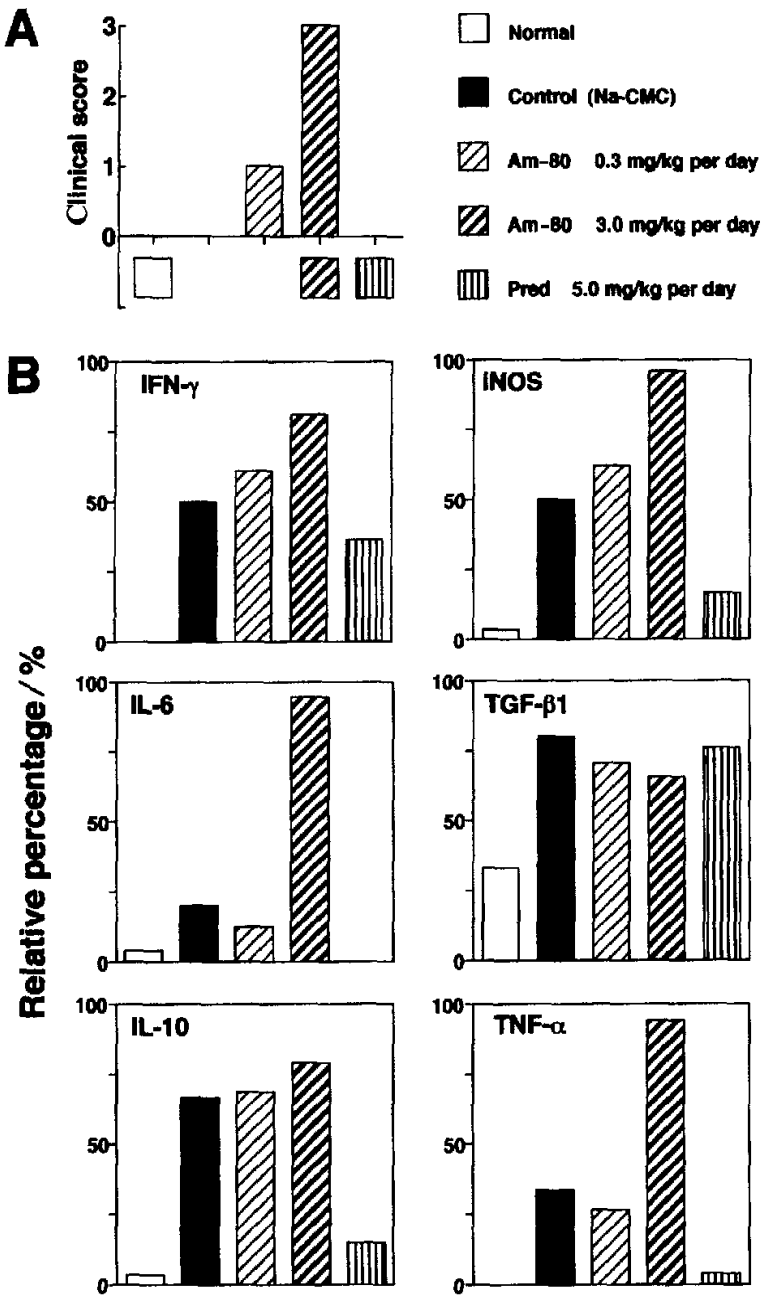

Fig 5. Effects of Am-80 and Pred on the mRNA expression of cytokines and iNOS in the spinal cord of DA rats 18 d after immunization. Am-80 and Pred were administered *po* daily for 12 d. A. Clinical score of each group tested. B. Changes in mRNA levels of different cytokines and iNOS are represented as the relative percentage compared with that of G3PDH. Each value represents the mean obtained from 3 – 4 samples.

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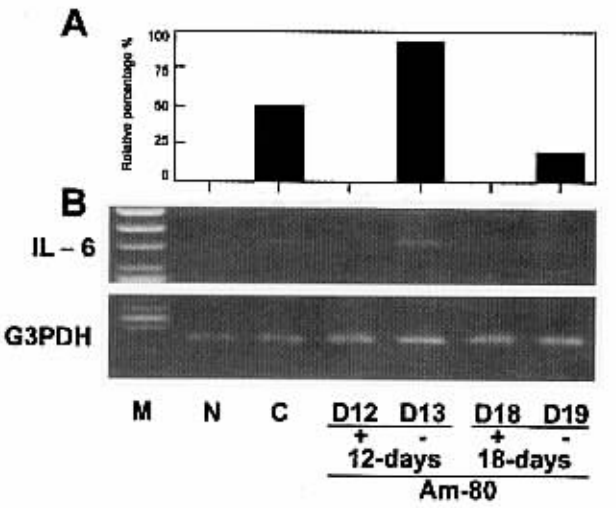

Fig 6. Effects of Am-80 on IL-6 mRNA expression in the spinal cords of DA rats during EAE development. Am-80 was administered *po* once a day for 12 or 18 d as indicated. A. Relative percentage of IL-6 mRNA levels when compared to G3PDH; B. Illustration of RT-PCR production. Fig A and B use the same abscissa as follows: M: Marker 5, N: Normal animals; C: Control animals; D 12 and D 18: 12 d and 18 d after immunization with Am-80 treated for 12 or 18 d, respectively; D 13 and D 19: 13 and 19 d after immunization with Am-80 treated for 12 or 18 d, respectively. Each value represents mean obtained from 3 – 4 samples.

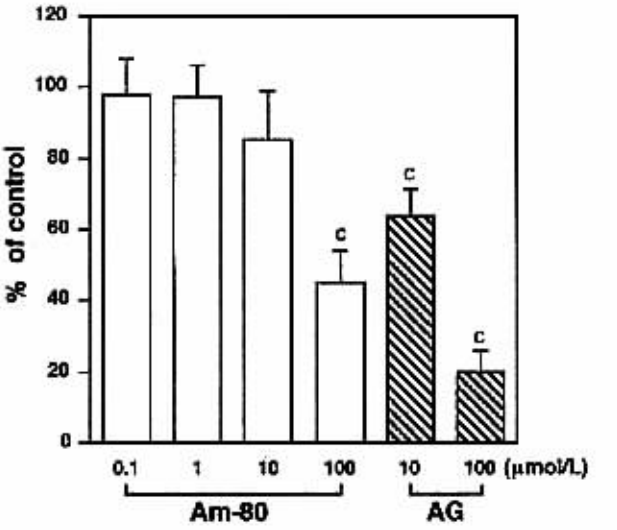

Fig 7. Effects of Am-80 (0.1 – 100 μmol/L) and aminoguanidine (AG, 10 – 100 μmol/L) on nitrite production from cultured RAW 264.7 cells induced by LPS(100 mg/L) plus IFN-γ (10 kU/L). Each value represents $\bar{x} \pm s$ obtained from multiple samples from three or more cell preparations. $^cP < 0.01$ *vs* control.

after cessation of Am-80 treatment.

Am-80 inhibited the DTH response during the treatment (on d 11) but did not inhibit the DTH response after treatment cessation suggesting that Am-80 inhibited the cell-mediated response.

In the present experiment, iNOS mRNA expression levels in the spinal cord changed with the severity of symptoms in both the control group and the Am-80 treated group, indicating that NO is involved in the inflammatory progression. Am-80 dose-dependently inhibited iNOS mRNA expression and this inhibition correlated with its effect on the disease. Since selective iNOS inhibitors appear to be effective for EAE treatment, we speculated that the effect of Am-80 on EAE might be mediated by iNOS mRNA. We thus tested this by examining the effect of Am-80 on iNOS mRNA expression and NO production from cultured RAW264.7 cells induced by LPS plus IFN-γ. However, *in vitro* data were inconclusive. Only a high concentration (100 mmol/L) of Am-80 significantly inhibited nitrite production, and this did not appear in the plasma of animals treated with the highest dose of Am-80 (3.0 mg/kg). At the same time, the inhibitory potency of AG on iNOS is higher than that of Am-80, but the dosage of AG needed to produce significant effects in rats is at least 200 mg/kg (sc or ip) daily[26,27], much higher than that of Am-80. These data show that the inhibitory effect of Am-80 on iNOS mRNA expression is not due to direct action of Am-80 on iNOS production, and is probably due to inhibition of the symptoms or other stimulators such as some cytokines[5,17,28].

Interestingly, 20 days after immunization and 18 days of treatment with Am-80, animals still developed EAE disease. In the relapsing EAE model, the relapsing response usually appears about 20 days after immunization with markedly weak severity compared with the first response. In this experiment, the relapsing response did not appear even if the observation was prolonged to 35 and 45 days after immunization when the animals were treated for 12 d and 18 d with Am-80. Although it is not clear how the immunity was stored for such long time and how disease was subsequently triggered, our findings indicate that IL-6 plays an initial role in EAE development.

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选择性白介素-6 抑制剂 Am-80 对 DA 大鼠实验性自身免疫性脑炎的影响

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关键词 变应性脑脊髓炎; Am-80; 白介素-6; 白介素-10; 转化生长因子 β; 肿瘤坏死因子; 一氧化氮合酶

目的: 研究选择性白介素(IL)-6 抑制剂 Am-80 对实验性自身免疫性脑炎(EAE)的影响, 并分析 IL-6 在 EAE 发病过程中的作用. **方法**: 诱导大鼠 EAE 模型, 半定量 RT-PCR 检测大鼠脊髓中细胞因子mRNA 表达, 培养 RAW264.7 细胞测定一氧化氮 (NO). **结果**: Am-80 (1.0, 3.0 mg/kg, ig × 12 d)能抑制髓鞘质碱性蛋白引起的 DA 大鼠 EAE 症状, 但高剂量 Am-80 并不能完全抑制 EAE 发病, 而只能延缓 EAE 发作时间, 停止给药后 EAE 病症仍然出现并有症状增强的现象; 延长 Am-80 的给药时间(18 d), EAE 仍然会在停止用药后出现. RT-PCR 证明 12 d Am-80 给药能抑制 EAE 大鼠脊髓中 IFN-γ, IL-6, IL-10, TGF-β1, TNF-α 和 iNOS 的 mRNA 表达水平, 但停药后一天(d 13)其它因子没有变化而 IL-6 mRNA 水平上升; 进一步采用 DA 大鼠 EAE 模型证明: 分别给予 12 d 和 18 d Am-80 后, IL-6 mRNA 表达水平的上升出现在停药后一天的 EAE 大鼠脊髓中. **结论**: IL-6 可能是 EAE 发病过程中的一个关键因素.

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