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The role of lipopolysaccharide injected systemically in the reactivation of collagen-induced arthritis in mice

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1 We investigated the role of bacterial lipopolysaccharide (LPS) in the reactivation of autoimmune disease by using collagen-induced arthritis (CIA) in mice in which autoimmunity to the joint cartilage component type II collagen (CII) was involved.

2 CIA was induced by immunization with CII emulsified with complete Freund's adjuvant at the base of the tail (day 0) followed by a booster injection on day 21. Varying doses of LPS from *E. coli* were i.p. injected on day 50.

3 Arthritis began to develop on day 25 after immunization with CII and reached a peak on day 35. Thereafter, arthritis subsided gradually but moderate joint inflammation was still observed on day 50. An i.p. injection of LPS on day 50 markedly reactivated arthritis on a dose-related fashion. Histologically, on day 55, there were marked oedema of synovium which had proliferated by the day of LPS injection, new formation of fibrin, and intense infiltration of neutrophils accompanied with a large number of mononuclear cells. The reactivation of CIA by LPS was associated with increases in anti-CII IgG and IgG2a antibodies as well as various cytokines including IL-12, IFN-γ, IL-1β, and TNF-α. LPS from *S. enteritidis, S. typhimurium*, and *K. neumoniae* and its component, lipid A from *E. coli* also reactivated the disease. Polymyxin B sulphate suppressed LPS- or lipid A-induced reactivation of CIA.

4 These results suggest that LPS may play an important role in the reactivation of autoimmune joint inflammatory diseases such as rheumatoid arthritis in humans.
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Keywords: Lipopolysaccharide; collagen-induced arthritis; cytokines; autoimmune disease; rheumatoid arthritis

Abbreviations: CIA, collagen-induced arthritis; CII, type II collagen; LPS, lipopolysaccharide; PMB, polymyxin B sulphate; RA, rheumatoid arthritis

Introduction

Lipopolysaccharide (LPS) is a biologically unique substance produced by Gram-negative bacteria. LPS activates B cells non-specifically, resulting in marked production of polyclonal antibodies (Dziarski, 1982). LPS also plays a role in the secretion of various mediators including IL-12 and IFN-γ involved in cellular immune responses (Fong & Mosmann, 1989; Panina-Bordignon *et al*., 1997). Therefore, some studies demonstrated the role of LPS in diseases in which autoimmune responses were involved. For instance, injection of LPS was followed by augmentation of autoimmune nephritis in BXSB, MRL/n, and NZW mice which was associated with increased deposition of pathogenic immune complexes in the microcirculation (Granholm & Cavallo, 1991; Hang *et al*., 1983). The endotoxin also enhances adoptive transfer of experimental allergic encephalomyelitis in rats by lymphoid cells sensitized with myelin basic protein (Hanada *et al*., 1989). However, few studies clearly demonstrated the role of LPS in autoimmune arthritis.

Collagen-induced arthritis (CIA) in mice is an experimental model of autoimmune diseases induced by immunization with type II collagen (CII) (Courtenay *et al*., 1980). Some clinical and histological features of CIA resemble those of rheumatoid arthritis (RA) in humans (Trentham, 1982; Stuart *et al*., 1982b). It has been shown that both cellular and humoral immune responses to CII are involved in the pathogenesis of CIA. For instance, the disease can be passively transferred to naive recipients by IgG antibodies specific for CII and their isotype IgG2a (Stuart *et al*., 1982a; Hirofuji *et al*., 1985). Lymphoid cells from animals immunized with CII (Trentham *et al*., 1978) and CII-specific T cell lines and clones (Holmdahl *et al*., 1985) also transmit the disease.

In the present study, we show that an i.p. injection of LPS from *E. coli*, *S. enteritidis*, *S. typhimurium* and *K. neumoniae*, and the LPS active site lipid A from *E. coli* reactivated CIA. We also show that the reactivated arthritis was associated with increased production of anti-CII IgG and IgG2a antibodies as well as varying kinds of cytokines including IL-12, IFN-γ, IL-1β, and TNF-α, suggesting that LPS plays a role in the exacerbation of the autoimmune joint inflammation.

Methods

Animals

Male DBA/1J mice, 8–9 weeks of age, were used in all experiments. The mice were bred in the animal breeding unit of Saga Medical School, Saga, Japan. They were maintained in a temperature- and light-controlled environmental with free access to standard rodent chow and water.

Induction of collagen-induced arthritis (CIA)

To induce CIA, 1 mg of type II collagen (CII) extracted from native calf articular cartilage (Funakoshi Co., Tokyo, Japan)

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was dissolved in 1 m of 0.1 N acetic acid and emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI, U.S.A.) (Yoshino, 1998a). One hundred microliters of the emulsion containing 50 μg of CII was injected s.c. into the base of the tail (day 0). Twenty-one days later, the animals were given a booster injection of the same amount of the emulsion at the same site. In some experiments, on day 50, 100 μg of CII dissolved in 100 μl of 0.005 N acetic acid was i.p. injected to further stimulate CII-specific immune response. To evaluate the severity of arthritis, the lesions of the four paws were each graded from 0–3 according to the increasing extent of erythema and oedema of the periarticular tissue as described elsewhere (Yoshino & Cleland, 1992). The maximum possible score is 12.

Administration of LPS

LPS from *E. coli* 011:B4 (Difco) was used in all experiments. Varying doses of LPS were dissolved in 100 μl of sterile, pyrogen-free saline and injected i.p. on day 50. As a control, 100 μl of saline alone was given on the same day. In some experiments, LPS from *S. enteritidis, S. typhimurium* (Difco), and *K. neumoniae* (Sigma Chemical Co., St. Louis, MO, U.S.A.) and lipid A from *E. coli* K12D31m4 (Funakoshi Co., Tokyo, Japan) were also i.p. administered.

Histology

Mice were killed on days 50 (immediately before administration of LPS) and 55. Hindpaws were amputated, fixed in 4% formalin, and decalcified (Yoshino *et al*., 1991). The tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin.

Measurement of antibodies to CII

Mice were killed on day 65 and sera collected were heat inactivated at 56°C for 30 min. Anti-CII IgG and IgG2a antibodies were measured by using an ELISA (Yoshino & Ohsawa, 1997). In brief, 96-well flat-bottomed microtiter plates were incubated with 100 μl well^{-1} of CII (100 μg ml^{-1}) at 37°C for 1 h and washed three times with PBS containing 0.05% Tween 20. The wells were then blocked by incubation with 100 μl of PBS containing 1% ovalbumin (Sigma) at 37°C for 1 h. After washing, the plates were incubated with 100 μl of a 1 : 600 dilution of each serum sample at 37°C for 30 min. The plates were washed, and 100 μl well^{-1} of a 1 : 1000 dilution of rat anti-mouse IgG or IgG2a labelled with alkaline phosphatase (Pharmigen, San Diego, CA, U.S.A.) was added and incubated at 37°C for 1 h. After washing, 100 μl of 3 mM of p-nitrophenylphosphate (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was added per well and the plates were incubated in the dark at room temperature for 15 min. The absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at $OD_{405} \pm$s.e.mean.

Measurement of cytokines

Spleens were removed on day 53 and cell suspensions were prepared. Erythrocytes in the cells were lysed with Tris-NH_4Cl. A total 5×10^6 cells in 1 ml of RPMI 1640 (Flow Laboratories, Inc., McLean, VA, U.S.A.) containing 1 mM glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 1% heat inactivated autologous mouse serum were cultured in 24-well tissue culture plates with 50 μg ml^{-1} CII (Yoshino, 1998b). Forty-eight hours later, supernatants were harvested and stored at −70°C until assayed. Cytokine production was quantified by ELISA. The ELISA kits for IL-12, IFN-γ, IL-1β, and TNF-α were purchased from Funakoshi Co. (Tokyo, Japan).

Administration of polymyxin B sulphate (PMB)

Fifty micrograms of PBM (Sigma) dissolved in 100 μl of sterile, pyrogen-free saline was mixed with 5 μg of LPS or 2 μg of lipid A and i.p. injected on day 50.

Treatment with anti-IFN-γ monoclonal antibody (mAb)

Anti-IFN-γ-mAb produced by the hybridoma R4-6A2 (American Type Culture Collection, Rockville, MD, U.S.A.) was precipitated by ammonium sulphate from ascitic fluid of SCID mice inoculated with the cells and purified with a protein G Sepharole 4 FF column (Pharmacia Biotec, Tokyo, Japan) (Yoshino, 1998a). The protein content was quantified by absorbance measurement at 280 nm. Purified anti-IFN-γ mAb (500 μg) dissolved in 0.1 ml saline was injected i.p. on day 50, immediately before injection of LPS, and then daily from days 51–55. The same amount of anti-IFN-γ mAb alone was also i.p. injected on the same days.

Statistics

To analyse data statistically, the Mann-Whitney *U*-test was used as one of non-parametric statistical methods since sample sizes in our experiments were small; therefore, the normality obtained was poor.

Results

The role of LPS in the reactivation of CIA

Mice immunized with CII had signs of arthritis on day 25 and the joint inflammation reached a peak on day 35 (Figure 1). Thereafter, the joint inflammation decreased gradually by day 50. Injection of LPS on day 50 resulted in marked reactivation of CIA. The reactivation of the disease by LPS was already observed on day 51 and reached a peak on day 55, followed by a relatively rapid decline by day 70. Not only already affected but also unaffected joints flared up following injection of the

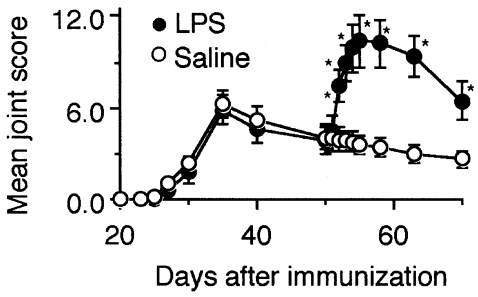

Figure 1 Reactivation of CIA by LPS. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. The severity of arthritis was determined on days 20, 23, 25, 27, 30 and 35, 40, 50, 51, 52, 53, 54, 55, 58, 63 and 70. Five micrograms of LPS was i.p. injected on day 50. Saline was administered as a control on the same days. Bars show the mean ± s.e.mean of 12 mice. *$P<0.05$ versus saline, Mann-Whitney U-test. Data are representative of three experiments.

endotoxin. Injection of saline on day 50 failed to reactivate the joint inflammation. Administration of varying doses of LPS showed that the reactivation of CIA was dose-dependent (Figure 2).

Histologically, on day 50 before injection of LPS, there was moderate proliferation of synovium with cell infiltrate in which mononuclear cells predominated (Figure 3A). Administration of LPS on day 50 was followed by marked oedema of the synovium, new formation of fibrin, and intense infiltration of neutrophils accompanied with a large number of mononuclear cells on day 55 (Figure 3B), while mice administered with saline showed histologic changes similar to those shown in Figure 3A (not shown).

The effect of LPS on anti-CII IgG and IgG2a antibody production

Since IgG and its isotype, IgG2a antibodies to CII play an important role in CIA (Stuart *et al*., 1982a; Hirofuji *et al*., 1985), production of these antibodies was examined after administration of LPS. As shown in Figure 4, injection of the endotoxin was followed by increases in anti-CII IgG and IgG2a antibodies, although the increase in anti-CII IgG2a appeared to be greater than that in anti-CII IgG.

The effect of LPS on cytokine secretion

To examine whether the reactivation of CIA by LPS was associated with secretion of cytokines, IL-12, IFN-γ, IL-1β, and TNF-α were measured on day 53. The results showed that administration of the endotoxin increased the levels of all these cytokines in a dose-related manner (Table 1). The increase rates of the secretion of IL-12, IFN-γ, IL-1β, and TNF-α by 5 μg of LPS were 1123, 746, 1198, and 1580%, respectively.

The role of varying types of LPS and lipid A in the reactivation of CIA

LPS from other Gram-negative bacteria and lipid A from *E. coli* were also used to test their ability to reactivate CIA. As shown in Figure 5, administration of all types of LPS from *S. enteritidis, S. typhimurium*, and *K. neumoniae* resulted in the reactivation of joint inflammation and the extent of the reactivation was similar to that caused by the endotoxin from

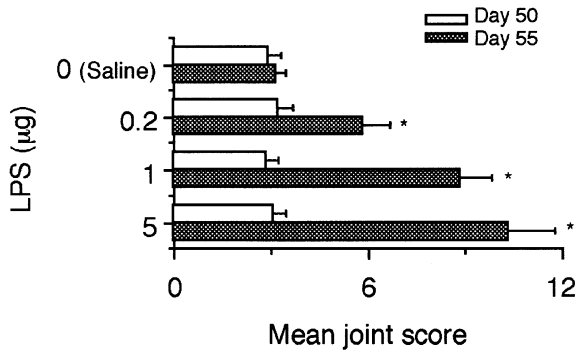

Figure 2 Dose-related reactivation of CIA by LPS. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. On day 50, saline and the indicated doses of LPS were i.p. injected. The severity of arthritis was determined on day 50, i.e. immediately before administration of LPS and on day 55. Bars show the mean ± s.e.mean of ten mice. $*P<0.05$ versus saline, Mann-Whitney U-test. Data are representative of three experiments.

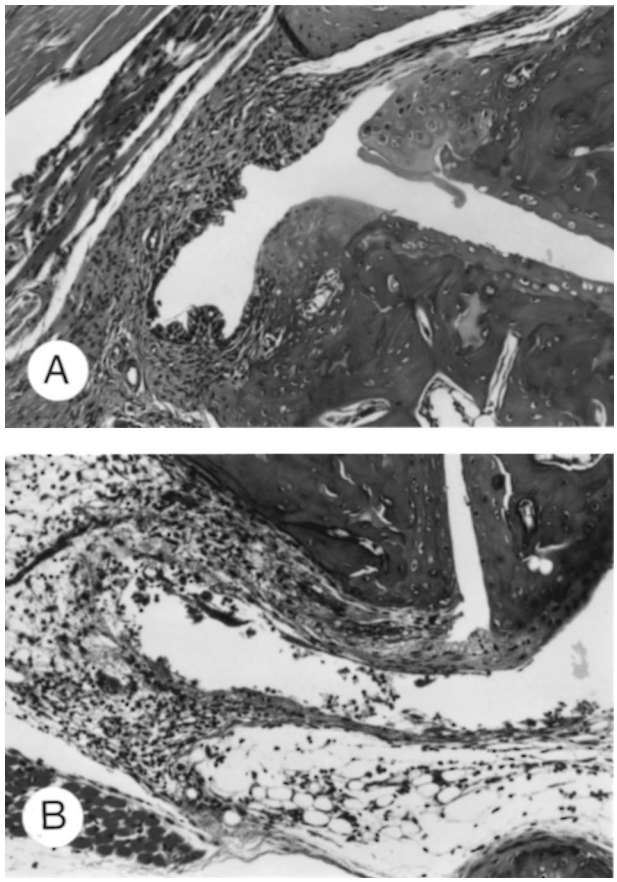

Figure 3 Histologic changes in tarsal joints of mice with CIA following administration of LPS. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. Saline or 5 μg of LPS was i.p. injected on day 50. Tarsal joints were histologically examined on day 50, i.e. immediately before injection of LPS (A) and on day 55 after injection of LPS (B). Haematoxylin and eosin stained, ×200 (original magnification).

Table 1 Secretion of cytokines in mice treated with LPS

	Cytokine (pg ml^{-1})							
LPS	*IL-12*		*IFN-γ*		*IL-1β*		*TNF-α*	
(μg)	*day 50*	*day 53*	*day 50*	*day 53*	*day 50*	*day 53*	*day 50*	*day 53*
0 (Saline)	462 ± 37	421 ± 53	358 ± 42	332 ± 27	530 ± 44	488 ± 35	376 ± 42	429 ± 53
0.2	438 ± 35	1420 ± 218*	294 ± 37	759 ± 61*	486 ± 52	2283 ± 174*	402 ± 35	3165 ± 472*
1	418 ± 46	3917 ± 416*	326 ± 25	2038 ± 348*	515 ± 39	5190 ± 472*	417 ± 36	5827 ± 328*
5	384 ± 52	4643 ± 382*	345 ± 40	2476 ± 172*	522 ± 40	5846 ± 418*	388 ± 30	6779 ± 582*

Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. On day 50, the indicated doses of LPS were i.p. injected. The secretion of IL-12, IFN-γ, IL-1β, and TNF-α from spleen cells were determined on day 50, i.e. immediately before injection of LPS and on day 53. Values are the mean ± s.e.mean of quadruplicate samples from culture supernatants of cells pooled from five mice. $*P<0.05$ versus saline, Mann-Whitney *U*-test. Data are representative of three experiments.

E. coli. Lipid A from *E. coli* was also active in exacerbating CIA significantly.

The effect of PMB on LPS- or lipid A-induced reactivation of CIA

To investigate whether PMB which neutralizes LPS and lipid A (van Miert *et al*., 1997) can suppress the reactivation of CIA by these bacterial cell wall components, the antibiotic was mixed with LPS or lipid A before injection on day 50. The results showed that PMB markedly blocked the reactivation of joint inflammation by these substances (Figure 6).

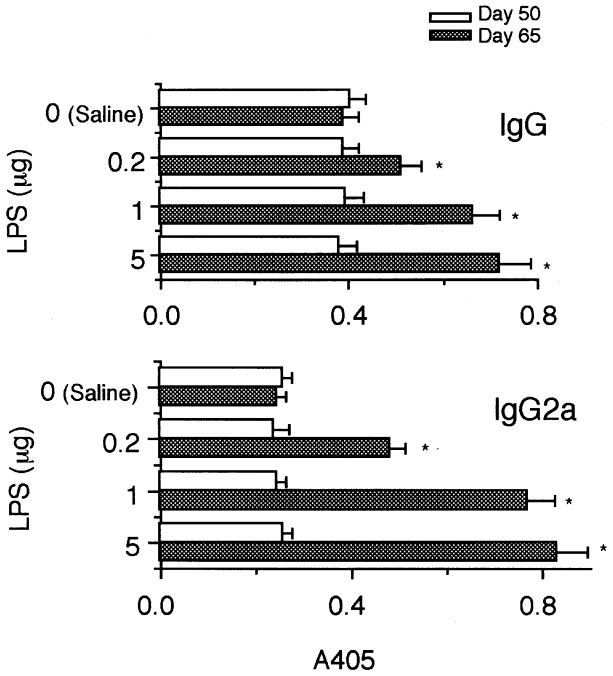

Figure 4 Enhancement of anti-CII antibody production by LPS. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. On day 50, saline and the indicated doses of LPS were i.p. injected and the serum levels of anti-CII IgG and IgG2a antibodies were determined on day 50, i.e. immediately before administration of LPS and on day 65. Bars show the mean ± s.e.mean of six mice. *$P<0.05$ versus saline, Mann-Whitney U-test. Data are representative of three experiments.

The effect of anti-IFN-γ mAb on LPS-induced reactivation of CIA

The role of IFN-γ involved in TH1 responses (Fong & Mosmann, 1989) in LPS-induced reactivation of CIA was investigated by using anti-IFN-γ mAb. As shown in Figure 7, the administration of LPS plus anti-IFN-γ mAb significantly diminished the enhancement of the severity of arthritis by the injection of LPS alone. The rates of increase in the severity of joint inflammation in mice treated with LPS plus anti-IFN-γ mAb and LPS alone were 83 and 257%, respectively. The administration of anti-IFN-γ mAb alone failed to affect the ongoing disease. Soluble CII alone injected on day 50 significantly reactivated CIA with the increase rate 54% in the severity of arthritis.

Discussion

In many autoimmune diseases including rheumatoid arthritis, remission and recurrence are often repeatedly observed. However, the cause of the recurrence as well as the mechanism of the remission are not well clarified at present. In the present study, we showed that bacterial LPS might play a role in the recurrence of autoimmune arthritis since systemic injection of the endotoxin from *E. coli* as well as from *S. enteritidis, S. typhimurium*, and *K. neumonia* markedly reactivated CIA in mice. The LPS active site lipid A was also effective in stimulating the existing joint inflammation. The reactivation of CIA by LPS and lipid A was blocked by PMB that neutralized these Gram negative bacterial cell wall components (van Miert *et al*., 1987), supporting the role of LPS and lipid A in the recurrence of joint inflammation. The reactivation of CIA by LPS was also confirmed by histologic changes in joints of LPS-treated mice showing marked oedema of synovium which had proliferated by the day of the endotoxin challenge, new formation of fibrin, and infiltration of many neutrophils accompanied with mononuclear cells, while control untreated animals failed to have such histologically acute inflammatory features.

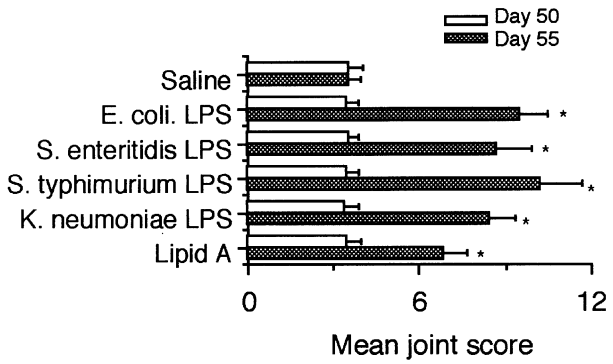

Figure 5 Reactivation of CIA by varying types of LPS and lipid A. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. On day 50, saline, 5 μg of LPS from *E. coli, S. enteritidis, S. typhimurium*, and *K. peumoniae*, and 2 μg of lipid A from *E. coli* were i.p. injected. The severity of arthritis was determined on day 50, i.e. immediately before administration of LPS and on day 55. Bars show the mean ± s.e.mean of eight mice. *$P<0.05$ versus saline, Mann-Whitney U-test. Data are representative of two experiments.

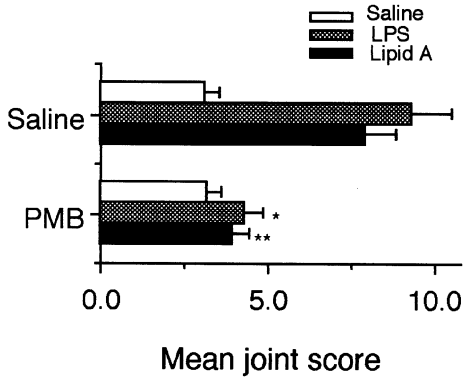

Figure 6 Suppression by polymyxin B of LPS-induced reactivation of CIA. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. On day 50, 100 μl of saline alone or 100 μl of saline containing 50 μg of polymyxin B sulphate (PMB) was mixed with saline, 5 μg of LPS, and 2 μg of lipid A and then i.p. injected on day 50. The severity of arthritis was determined on day 55. Bars show the mean ± s.e.mean of ten mice. *$P<0.05$ versus saline- and LPS-treatment; **$P<0.05$ versus saline- and lipid A-treatment, Mann-Whitney U-test. Data are representative of two experiments.

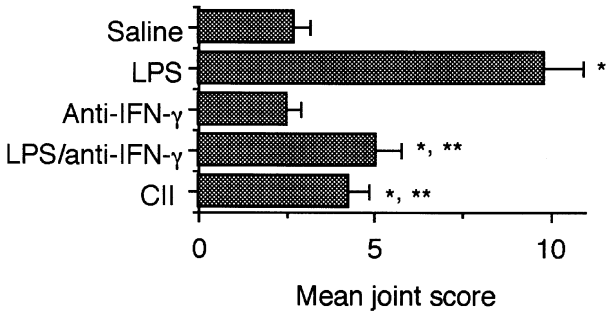

Figure 7 Effect of anti-IFN-γ mAb on reactivation of CIA by LPS. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Methods. On day 50, saline, 5 μg of LPS alone, 500 μg of anti-IFN-γ mAb alone, 5 μg of LPS plus 500 μg of anti-IFN-γ mAb, or 100 μg of CII was i.p. injected. Injection of anti-IFN-γ mAb was continued daily from days 51 to 55. The severity of arthritis was determined on day 60. Bars show the mean$\pm$s.e.mean of eight mice. *$P<0.05$ versus saline; **$P<0.05$ versus LPS alone, Mann-Whitney U-test. Data are representative of two experiments.

There are a number of studies demonstrating a role for LPS in autoimmune diseases. For instance, it was previously shown that the injection of LPS enhanced autoimmune nephritis in BXSB, MRL/n, or NZW lupus-prone mice (Granholm & Cavallo, 1991; Hang *et al*., 1983) and adoptive transfer of experimental autoimmune encephalomyelitis (Hanada *et al*., 1989). It was also demonstrated that LPS acted as an adjuvant to induce autoimmune uveitis (Yokochi *et al*., 1993) and autoimmune myocarditis in rats (Kato *et al*., 1993). However, few studies clearly showed that LPS has ability to reactivate existing autoimmune arthritis.

Although the precise mechanism of the reactivation of CIA by LPS is currently unclear, the increased production of in anti-CII IgG and the isotype IgG2a antibodies following treatment with the endotoxin may have in part contributed to the reactivation of the disease because it was previously demonstrated that IgG, especially the IgG2a antibodies to CII played a critical role in CIA (Stuart *et al*., 1982a; Hirofuji *et al*., 1985). The marked production of anti-CII IgG2a antibodies by LPS may be due to the augmented secretion of IFN-γ since this Th1 cytokine plays a role in production of this isotype of antibody (Snapper & Paul, 1987; Finkelman *et al*., 1988). Furthermore, since LPS does not normally activate T cells directly, but stimulates non-lymphocytes such as macrophages known to be a IL-12-producing cell (Verhasselt *et al*., 1997) and because this cytokine plays a role in the secretion of IFN-γ, the enhanced secretion of IFN-γ may be due to the increase in IL-12 in mice injected with the endotoxin.

The exacerbation of CIA by LPS may also be explained by the increased secretion by the endotoxin of other cytokines including IL-1β and TNF-α which are involved not only in immune responses but also in inflammation itself (Arend & Dayer, 1995; Brennan *et al*., 1992). Stimpson *et al*. (1987) demonstrated that arthritis induced by the toxic effect of peptidoglycan-polysaccharide polymers injected intra-articularly in rats was reactivated by a systemic injection of LPS. It is also likely that anti-CII antibodies and the above proinflammatory mediators acted synergistically to reactivate arthritis. Terato *et al*. (1995) showed that injection of LPS reduced the amount of anti-CII monoclonal antibodies required for the induction of joint inflammation, suggesting the synergistic effect of LPS on arthritis.

The involvement of proinflammatory cytokines as well as CII-specific immune responses in the reactivation of CIA by LPS was also supported by the experiments in which anti-IFN-γ mAb was used to neutralize the cytokine so that antigen-specific Th1 responses were expected to be suppressed (Fong & Mossmann, 1989). We previously demonstrated that CIA appeared to be a Th1-dominant disease (Yoshino, 1998a). It is of note that proinflammatory cytokines might have more contributed to the flare up than CII-specific Th1 immune responses since the treatment with LPS plus anti-IFN-γ-mAb was followed by an increase in the severity of joint inflammation up to 83%, while the injection of LPS alone resulted in a greater increase (257%). Furthermore, only 54% increase in the severity was seen in mice injected with soluble CII alone. The result showing no difference in the severity of CIA between mice treated with saline- and anti-IFN-γ alone on day 50 suggests that Th1 responses have not been critically involved in the ongoing disease at that time.

Aoki *et al*. (1996) reported that patients with RA had significantly increased titers of antibodies against *E. coli* in the sera and synovial fluids compared with control healthy subjects, indicating that the patients appeared to be more sensitized with the enteric bacteria. By using immunoblot analysis, they also found a ladder-like banding pattern equivalent to enterobacterial common antigen associated with LPS. Heumann *et al*. (1995) showed high levels of LPS binding protein in sera and in synovial fluids in RA patients. We recently found that LPS administered orally was detected in blood (unpublished data), suggesting the endotoxin from intestinal flora may cross the mucosa and be distributed systemically. In addition, CII is a major component of cartilage and anti-CII IgG antibodies are seen in sera from subjects with RA (Gay *et al*., 1980; Stuart *et al*., 1983). Furthermore, CII and anti-CII antibody complexes are present in sera from the patients (Claque & Moore 1984). Taken together, these findings suggest that LPS may play a role in the recurrence of RA, although the definite role of anti-CII antibodies in the disease has not been established.

In summary, the systemic injection of LPS resulted in the reactivation of CIA in mice that was associated with the increased production of anti-CII IgG and IgG2a antibodies as well as the enhanced secretion of cytokines including IL-12, IFN-γ, IL-1β, and TNF-α. Thus, LPS may play a role in the exacerbation of autoimmune diseases.

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