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Oral Administration of Lipopolysaccharide Exacerbates Collagen-Induced Arthritis in Mice

Shin Yoshino, Eizaburo Sasatomi, Yoki Mori, and Masaru Sagi

We investigated whether oral administration of LPS exacerbated collagen-induced arthritis (CIA) in mice, which was an experimental model of autoimmune disease. CIA was induced by s.c. injection of type II collagen emulsified with CFA into the base of the tail (day 0) followed by a booster injection on day 21. To examine the ability of LPS to exacerbate CIA, varying doses of LPS were orally administered on day 50. The results showed that administration of LPS was followed by reactivation of CIA in a dose-related fashion. Histologically, on day 55 there were marked edema of synovium proliferated by day 50, new formation of fibrin, and intense infiltration of neutrophils accompanied with a large number of mononuclear cells. Severe destruction of cartilage and subchondral bone was also observed on day 70. The reactivation of CIA by oral administration of LPS was associated with increase in anti-type II collagen IgG and IgG2a Abs as well as varying kinds of cytokines including IL-12, IFN-γ, IL-1β, and TNF-α. Polymyxin B sulfate given either orally or i.v. suppressed the recurrence of CIA. Increased amounts of LPS were found in sera of mice given the endotoxin orally. LPS from Salmonella enteritidis, Salmonella typhimurium, and Klebsiella pneumoniae and its component, lipid A from Escherichia coli, also reactivated the disease. These findings suggest that LPS from intestinal bacteria may play a role in the exacerbation of autoimmune joint inflammation. The Journal of Immunology, 1999, 163: 3417–3422.

Collagen-induced arthritis (CIA) is an experimental model of autoimmune disease that can be induced in mice (1), rats (2), and primates (3) by immunization with type II collagen (CII). Many features of CIA resemble those of rheumatoid arthritis in humans (4, 5). 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 Abs specific for CII and their isotype IgG2a (6, 7). Lymphoid cells from animals immunized with CII (8) and CII-specific T cell lines and clones (9) also transmit the disease.

LPS is a component of the Gram-negative bacterial cell wall that activates B cells, resulting in marked production of polyclonal Abs (10). LPS also plays a role in the secretion of various mediators including IL-12 and IFN-γ involved in cellular immune responses (11, 12). Accordingly, a number of studies demonstrated the role of LPS in some diseases in which autoimmune responses were involved. For instance, a systemic injection of LPS was followed by augmentation of autoimmune nephritis in BXSB, MRL/n, or NZW mice that was associated with increased deposition of pathogenic immune complexes in the microcirculation (13). However, few studies demonstrated the role of LPS in the exacerbation of CIA, and more importantly, previous studies did not show the direct role of the endotoxin derived from the gut in autoimmune diseases despite the fact that an extremely large number of LPS-producing normal flora including Escherichia coli are present in intestinal tracts.

In the present study, we show that oral administration of LPS from E. coli, Salmonella enteritidis, Salmonella typhimurium, and Klebsiella pneumoniae and lipid A from E. coli resulted in exacerbation of CIA and the exacerbated arthritis was associated with increased levels of the endotoxin in serum, suggesting that LPS from enteric bacteria may play a role in the exacerbation of autoimmune joint inflammation.

Materials and Methods

Animals

Male DBA/1J mice, 6–9 wk 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 under clean conventional conditions with free access to standard rodent chow and water.

Induction of CIA

To induce CIA, 1 mg of CII extracted from native calf articular cartilage (Funakoshi, Tokyo, Japan) was dissolved in 1 ml of 0.1 M acetic acid and emulsified with an equal volume of CFA (Difco, Detroit, MI) (14). The emulsion (100 µl) 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. To evaluate the severity of arthritis, the lesions of the four paws were each graded from 0 to 3 according to the increasing extent of erythema and edema of the periarticular tissue as described elsewhere (15). The maximum possible score was 12.

Administration of LPS

LPS from E. coli 011:B4 (Difco) was used in experiments. Varying doses of LPS were dissolved in 0.2 ml of PBS and administered post orally (per os, p.o.) through a syringe fitted with an 18-gauge ballpoint needle on day 50 or 80. As a control, PBS was given on the same day. In some experiments, LPS from S. enteritidis, S. typhimurium (Difco), and K. pneumoniae (Sigma, St. Louis, MO) and lipid A from E. coli K12D31 m4 (Funakoshi) were also p.o. administered.

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Histology

Mice were killed on either on day 50 (immediately before administration of LPS) or on day 55 or 70. Hindpaws were amputated, fixed in 4% formalin, and decalcified. The tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin.

Measurement of Abs to CII

Mice were killed on day 65 and the sera collected were heat inactivated at 56°C for 30 min. Anti-CII IgG and IgG2a Abs were measured using an ELISA (16). In brief, 96-well flat-bottom microtiter plates were incubated with 100 μl/well of CII (100 μg/ml) 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% OVA (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 of a 1:1000 dilution of rat anti-mouse IgG or IgG2a labeled with alkaline phosphatase (PharMingen, San Diego, CA) was added and incubated at 37°C for 1 h. After washing, 100 μl of 3 mM p-nitrophenylphosphate (Bio-Rad, Richmond, CA) 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 OD405 ± SEM.

Measurement of cytokines

Spleens were removed on day 55, and cell suspensions were prepared. Erythrocytes in the cells were lysed with Tris-NH₄Cl. A total 5 × 10⁶ cells in 1 ml of RPMI 1640 (Flow Laboratories, McLean, VA) containing 1 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10⁻⁵ M 2-ME, and 1% heat inactivated autologous mouse serum were cultured in 24-well tissue culture plates either in medium alone or with 50 μg/ml CII (17). Forty-eight hours later supernatants were harvested and stored at −70°C until assayed. Cytokine production was quantified by ELISA.
The role of oral administration of LPS in the exacerbation of CIA

Signs of arthritis were observed on day 24 after immunization with CII (Fig. 1). All mice developed joint inflammation by day 30, and arthritis subsided relatively rapidly by day 50, although complete remission was not seen at least by day 100. The oral administration of 1 mg of LPS reached a peak on day 35. Thereafter, arthritis subsided gradually. The additional oral administration of LPS on day 80 was followed by another reactivation of joint inflammation. The exacerbation of CIA by LPS was seen in a dose-related fashion (Fig. 2).

The effect of oral administration of LPS on histologic changes in joints

Histological evaluation of joints of mice with CIA was done on day 50 (immediately before administration of LPS) and on days 55 and 70. On day 50, there were moderate proliferation of synovium, cell infiltration in which mononuclear cells predominated, and relatively weak destruction of cartilage (Fig. 3A). Oral administration of LPS was followed by marked edema of proliferated synovium, new formation of fibrin, and intense infiltration of neutrophils accompanied by a large number of mononuclear cells on day 55 (Fig. 3B), whereas mice given PBS orally showed histologic changes similar to those shown in Fig. 3A (data not shown). On day 70, severe destruction of cartilage and subchondral bone caused by many inflammatory cells was observed in LPS-treated mice (Fig. 3C). PBS-treated mice had moderate destruction of the joint components (Fig. 3D).

The effect of oral administration of LPS on anti-CII IgG and IgG2a Ab production

Because IgG and its isotype IgG2a Abs against CII are critically involved in the development of CIA (6, 7), the levels of these Abs were determined after oral administration of LPS. As shown in Fig. 4, significantly greater production of anti-CII IgG and IgG2a Abs was seen in mice given >0.3 and >0.1 mg of LPS, respectively, than those fed PBS. There was no difference in the levels of these Abs between PBS- and LPS-treated animals before administration of the endotoxin.

The effect of oral administration of LPS on the secretion of cytokines

To examine whether the exacerbation of CIA by orally given LPS was associated with secretion of cytokines, IL-12, IFN-γ, IL-1β, and TNF-α were measured on day 55. The results showed that all the cytokines were produced following administration of the endotoxin in a dose-dependent manner (Table I). The secretion of IL-12, IFN-γ, IL-1β, and TNF-α was 17.0, 8.0, 25.9, and 22.2 times greater, respectively, in mice given 1 mg of LPS than those fed PBS. No differences in the secretion of these cytokines between mice treated with LPS and PBS before administration of the endotoxin or PBS were observed.

Table I. Secretion of cytokines in mice given LPS orally

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>LPS (mg)</th>
<th>Day 50</th>
<th>Day 55</th>
<th>Day 50</th>
<th>Day 55</th>
<th>Day 50</th>
<th>Day 55</th>
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<tr>
<td>IL-12</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>0 (PBS)</td>
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<td>297 ± 31</td>
<td>322 ± 26</td>
<td>196 ± 28</td>
<td>273 ± 30</td>
<td>329 ± 43</td>
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<td>0.1</td>
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<td>320 ± 22</td>
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<td>223 ± 32</td>
<td>320 ± 41</td>
<td>294 ± 38</td>
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<td>342 ± 36</td>
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<tr>
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<td>345 ± 42</td>
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<td>205 ± 31</td>
<td>1476 ± 128*</td>
<td>346 ± 51</td>
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<td>302 ± 28</td>
<td>5464 ± 388*</td>
<td>232 ± 30</td>
<td>2195 ± 197*</td>
<td>370 ± 42</td>
<td>9365 ± 583*</td>
<td>328 ± 23</td>
<td>7874 ± 632*</td>
</tr>
</tbody>
</table>

* Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Materials and Methods. On day 50, the indicated doses of LPS were p.o. administered. The secretion of IL-12, IFN-γ, IL-1β, and TNF-α from spleen cells was determined immediately before administration of LPS and on day 55. Values are the mean ± SEM of quadruplicate samples from culture supernatants of cells pooled from five mice.

* p < 0.05 vs PBS, Mann-Whitney U test.
vs PBS, Mann-Whitney U test. Bars show the mean ± SEM of five mice. *, p < 0.05 vs PBS, Mann-Whitney U test.

FIGURE 6. Elevated levels of LPS in sera of mice given p.o. the endotoxin. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Materials and Methods. On day 50, the indicated doses of LPS were p.o. given to arthritic (open bar) or normal (hatched bar) mice. Two hours later the animals were killed and the amounts of LPS in sera were determined. The levels of LPS in sera before administration of the endotoxin were <2 pg/ml. Bars show the mean ± SEM of five mice. *, p < 0.05 vs PBS, Mann-Whitney U test.

The effect of PMB on the exacerbation of CIA by oral administration of LPS

To investigate whether PMB, which neutralizes LPS (19), can suppress the exacerbation of CIA by oral administration of LPS, Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Materials and Methods. On day 50, the indicated doses of LPS were p.o. or i.v. administered, respectively, immediately before p.o. administration of 1 mg of LPS. PBS was administered as a control. The severity of arthritis was determined on day 50, i.e., immediately before administration of LPS (open bar) and on day 60 (hatched bar). Bars show the mean ± SEM of eight –10 mice. *, p < 0.05 vs PBS, Mann-Whitney U test.

FIGURE 5. Suppression by PMB of exacerbation of CIA by oral administration of LPS. Mice were immunized with CII on day 0 followed by a booster injection on day 21 as described in Materials and Methods. On day 50, 5 mg and 50 µg of PMB were p.o. and i.v. administered, respectively, immediately before p.o. administration of 1 mg of LPS. PBS was administered as a control. The severity of arthritis was determined on day 50, i.e., immediately before administration of LPS (open bar) and on day 60 (hatched bar). Bars show the mean ± SEM of eight –10 mice. *, p < 0.05 vs PBS, Mann-Whitney U test.

Levels of LPS in sera of animals given the endotoxin orally

Because the preventive effect of PMB injected i.v. on the reactivation of CIA suggested that LPS given p.o. might cross the intestinal mucosa and enter the circulation, levels of the endotoxin in serum were determined 2 h after its p.o. administration. As shown in Fig. 6, increased amounts of LPS were detected in the sera of mice with arthritis as well as normal animals p.o. given 0.1, 0.3, and 1 mg of the endotoxin in a dose-related fashion.

The effect of p.o. vs i.v. administration of LPS on the exacerbation of CIA

The ability of LPS administered p.o. to exacerbate CIA was compared with that of the endotoxin given i.v. The results showed that as low as 0.1 µg of LPS injected i.v. was sufficient to reactivate joint inflammation significantly (Fig. 7). The extent of reactivation of CIA by i.v. injection of 0.3, 1, and 3 µg of LPS was approximately equal to those by p.o. administration of 0.1, 0.3, and 1 mg of the endotoxin, respectively.

The effect of varying types of LPS and lipid A on the exacerbation of CIA

LPS from other Gram-negative bacteria and lipid A from E. coli were also used to test their ability to induce exacerbation of CIA. As shown in Fig. 8, oral administration of all types of LPS from S. enteritidis and S. typhimurium, and K. pneumoniae reactivated joint inflammation significantly, and the extent of the reactivation was similar to that caused by the endotoxin from E. coli. Lipid A from E. coli was also active in exacerbating CIA significantly.

Discussion

The present study implies that LPS and its component, lipid A derived from intestinal bacteria, may play a role in the exacerbation of autoimmune arthritis because oral administration of the endotoxin from E. coli, as well as from S. enteritidis, S. typhimurium, and K. pneumonia and lipid A from E. coli after CIA had been subsided reactivated the joint inflammation. The reactivation of CIA by LPS was confirmed by histologic changes in joints showing marked edema of synovium proliferated by the day of administration of the endotoxin, new formation of fibrin, massive infiltration of neutrophils accompanied with a number of mononuclear cells, and advanced destruction of cartilage and subchondral bone.

There have been a number of studies demonstrating the effect of systemic injection of LPS on autoimmune disease. For instance, it was shown that injection of LPS enhanced autoimmune nephritis in BXSB, MRL/n, or NZW lupus-prone mice (13) and experimental autoimmune encephalomyelitis (20), although, to our knowledge, the effect of the endotoxin on the exacerbation of CIA was not examined previously. It was also shown that LPS played a role as an adjuvant in the induction of autoimmune uveitis (21), autoimmune myocarditis (22), and arthritis caused by mAbs to CII (23). These studies suggest the role of circulating LPS but do not
directly imply the role of the endotoxin from the gut in autoimmune disease.

The role of enteric bacteria in the onset of autoimmune disease was previously demonstrated by Murakami et al. (24) by using a model of autoimmune hemolytic anemia in anti-RBC autoantibody transgenic mice. For instance, they found that the transgenic mice failed to develop anemia when bred in germ-free conditions whereas they developed the disease when bred in a conventional environment. Furthermore, when the animals bred in germ-free conditions were transferred to the conventional condition or injected with LPS, they suffered from the autoimmune disease.

Not only p.o. administration but also i.v. injection of the anti-biotic PMB that neutralizes LPS by binding lipid A (19) significantly suppressed the exacerbation of CIA by the endotoxin given p.o., suggesting that intestinal LPS may cross the mucosa and be distributed systematically. In fact, significantly increased amounts of LPS were found in sera of mice given the endotoxin p.o. The direct role of LPS in the reactivation of CIA was confirmed by injecting i.v. the endotoxin as shown in the present study.

The precise mechanism by which CIA was exacerbated by LPS is unclear at present. However, increases in anti-CII IgG and the isotype IgG2a Abs following oral administration of the B cell activator appeared to have, at least in part, contributed to the reactivation of the autoimmune disease because these Abs, especially the IgG2a, played a critical role in CIA (6, 7). Marked production of anti-CII IgG2a Abs by LPS may be due to the enhanced secretion of IFN-γ observed in mice given the endotoxin because this Th1 cell-producing cytokine was involved in the isotype Ab production (25, 26). Furthermore, because LPS does not normally activate T cells directly, but stimulates nonlymphocytes such as macrophages producing IL-12 (27), which acts on T cells, the enhanced secretion of IFN-γ appeared to be due to the increase in IL-12 in the endotoxin-treated animals.

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 (28, 29). Stimpson et al. (30) demonstrated that arthritis induced by the toxic effect of peptidoglycan-polysaccharide polymers injected intraarticularly in rats was reactivated by a systemic injection of LPS.

It was previously shown that fever therapy was an effective therapy for rheumatoid arthritis (31, 32). Because LPS induces fever (33), the endotoxin may act as an anti-inflammatory agent. However, no suppression of CIA was seen in mice treated with LPS. Conversely, as already shown above, arthritis was markedly enhanced by its treatment. This result is probably due to the ability of LPS to provoke marked inflammatory responses, which overcomes that to suppress inflammation. In addition, LPS also have anti-inflammatory effects through its capacity to induce leukopenia (34, 35). However, the anti-inflammatory effects are observed only when the endotoxin is administered before induction of inflammation. Because, in our experiments, LPS was given after onset of CIA, anti-inflammatory effects of the substance appear to be not considered.

Aoki et al. (36) reported that patients with rheumatoid arthritis had significantly increased titers of Abs 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 Ag associated with LPS. Heumann et al. (37) showed high levels of LPS-binding protein in sera and in synovial fluids in patients with rheumatoid arthritis. These findings suggest that LPS may play a role in the autoimmune joint inflammatory disease.

In summary, oral administration of LPS resulted in the exacerbation of CIA in mice that was associated with increased production of anti-CII IgG and IgG2a Abs as well as enhanced secretion of cytokines including IL-12, IFN-γ, IL-1β, and TNF-α. The exacerbated joint inflammation appeared to be due to the absorption of the endotoxin from the gut. Thus, LPS from intestinal bacteria may be crucially involved in the reactivation of autoimmune joint inflammation such as rheumatoid arthritis, although no definite role of anti-CII Abs in the human disease has been established.

References


