Lack of cross tolerance between LPS and muramyl dipeptide in induction of circulating TNF-α and IL-6 in guinea pigs

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Roth, j oachim, Tunay Aslan, Birgit Störr, and Eugen Zeisberger. Lack of cross tolerance between LPS and muramyl dipeptide in induction of circulating TNF-α and IL-6 in guinea pigs. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1529–R1533, 1997.—In guinea pigs, lipopolysaccharide (LPS) from gram-negative bacteria and muramyl dipeptide (MDP) from gram-positive bacteria are potent inducers of systemic production of proinflammatory cytokines and fever. However, there is a striking difference between these two bacterial pyrogens in so far as repeated administration of LPS, but not of MDP, in short-term intervals induces tolerance by a progressive downregulation of the systemic cytokine network. In the present study, we investigated MDP-induced fever and the systemic release of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in LPS-tolerant guinea pigs in comparison with naive animals. Endotoxin tolerance was induced by repeated intramuscular injections of 20 µg/kg LPS at intervals of 3 days. In response to the last of five injections with LPS, systemic production of TNF-α and IL-6 as well as the development of a febrile response was abrogated almost completely. Those guinea pigs that had developed an LPS tolerance could, however, produce a response, including fever (7, 21, 23, 26, 27) and the production of cytokines in vitro (16, 28) and in vivo (23).

MATERIALS AND METHODS

Animals. This study was performed in 28 male guinea pigs with a body weight of 390 ± 5 g at the beginning of the experiments. The animals were housed in individual cages at 22°C and on a 12:12-h light-dark cycle (light off at 7:00 PM).

Surgery. At least 1 wk before the start of the experimental procedure, two groups of animals anesthetized with 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine were chronically implanted with intra-arterial catheters as described previously (22, 25). Two other groups of guinea pigs were intraperitoneally implanted with biotelemetry transmitters for measurement of abdominal temperature (see below).

Substances. Bacterial LPS (derived from Escherichia coli, O111:B4; Sigma Chemical, St. Louis, MO) was dissolved in saline

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In humans or experimental animals, injection with lipopolysaccharide (LPS), a component of the outer membrane of most gram-negative bacteria, results in the induction of the acute-phase response. Several components of the acute-phase response, such as fever (2, 24, 25, 27), anorexia (20), the fall in serum iron concentration (10), and even mortality (11) are progressively attenuated when LPS is administered repeatedly in short-term intervals, a phenomenon called endotoxin tolerance. This phenomenon has been known for about 50 years (3), and it has been suggested that tolerance may result from the limitation in the host’s capacity to mobilize a postulated “endogenous pyrogen” long before cytokines were identified as pyrogenic molecules (2). Decades later, several studies in vivo and in vitro provided evidence that the development of LPS tolerance is associated with a reduced production of cytokines (11, 14, 15, 24, 30), which are regarded as the endogenous mediators of the acute-phase response (13). Thus the attenuation of fever during the development of endotoxin tolerance strongly correlates with a reduction of circulating tumor necrosis factor (TNF)-α and interleukin (IL)-6 (24).

Several studies in vitro were performed to investigate the critical step within the LPS-induced signal pathway for cytokine formation, which is blocked after the induction of endotoxin tolerance. It has been demonstrated that a downregulation in the expression of the CD14 LPS receptor is not the mechanism for the induction of LPS tolerance because the expression of the CD14 receptor remained almost unchanged in peritoneal macrophages of rabbits adapted to LPS (18). The authors of that study concluded that the LPS-induced hyporesponsiveness resulted from changes in cellular elements distinct from the CD14 receptor. A strong reduction of LPS-induced intracellular protein phosphorylation was observed in murine peritoneal macrophages that were made tolerant to LPS (19). Finally, it was suggested that a transcriptional repressor molecule is responsible for the lack of LPS-induced transcription of cytokine genes in LPS-tolerant human monocytes (15). If such a blockade of the cytokine genes was the most important mechanism for the induction of LPS tolerance, could it then still be possible to activate cytokine formation by a stimulus distinct from LPS, such as muramyl dipeptide (MDP), the minimal immunoadjuvant structure of gram-positive bacterial cell walls (8)? This substance displays profound immunopharmacological activities and can be used as an inducer of several components of the acute-phase response, including fever (7, 21, 23, 26, 27) and the production of cytokines in vitro (16, 28) and in vivo (23).

If a production of cytokines (endogenous pyrogens) was possible in LPS-tolerant animals, a reasonable explanation for the reported lack of cross tolerance in the pyrogenic effects between LPS and MDP in rabbits (27) could be provided. Therefore, we used MDP to induce production of cytokines and fever in LPS-tolerant compared with naive guinea pigs.
sterile pyrogen-free 0.9% saline at a concentration of 100 µg/ml. An amount of 20 µg/kg was injected intramuscularly into the thigh muscle.

MDP (N-acetyl-

l-alanyl-d-isoglutamine; ICN Biomedicals, Aurora, OH) was dissolved in sterile pyrogen-free 0.9% saline at a concentration of 500 µg/ml. An amount of 100 µg/kg was used for intramuscular injections.

Experimental protocols. Experiment 1 was performed to investigate the MDP-induced production of cytokines in LPS-tolerant guinea pigs. A group of seven animals was injected with LPS five times at intervals of 3 days to induce endotoxin tolerance. In experiments 1, 3, and 5, single blood samples (0.6 ml) were slowly (within 1 min) collected 60 min before as well as 60 and 180 min after injection of LPS. Blood was drawn into a sterile syringe, put into a polypropylene tube, and immediately centrifuged. The plasma blood was stored at −70°C for later determination of cytokines. Three days after the last of five injections with LPS (in a state of endotoxin tolerance), the animals were injected with MDP. The MDP-induced production of TNF and IL-6 in LPS-tolerant guinea pigs was compared with the MDP-induced cytokine formation in a group of seven naive animals.

Experiment 2 was performed to evaluate MDP-induced fever in LPS-tolerant guinea pigs. Another group of seven animals, equipped with radiotelemetry transmitters, received repeated injections of LPS according to the time schedule described for experiment 1. Three days after the last of five injections with LPS (in a state of endotoxin tolerance), the guinea pigs were injected with MDP and the resulting febrile response was compared with the MDP-induced fever in a group of seven naive animals.

Time schedule and the intramuscular route of injection were elected to be able to compare our data with the results from our previous studies on the development of pyrogenic tolerance in guinea pigs (5, 24, 25).

Cytokine bioassays. The mouse fibrosarcoma cell line WEHI 164 subclone 13 (provided by Dr. Stephen Hopkins, University of Manchester, UK) was used to determine bioactive TNF levels (9). The assay was based on the dose-dependent cytotoxic effect of TNF-α on this cell line and was performed in 96-well microtiter plates with 50,000 actinomycin D-treated WEHI cells per well. The number of living cells incubated for 24 h with serial dilutions of biological samples or with different concentrations of TNF standard (code 88/532, National Institute for Biological Standards and Control, South Mimms, UK) was measured by use of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (12) with an enzyme-linked immunosorbent assay reader (MR 7000; Dynatech Laboratories, Denkendorf, Germany) at 550 nm.

Determination of IL-6 was performed by a bioassay based on the dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line (1). The B9 cells were kindly provided by Dr. Stephen Hopkins. The assay was performed in sterile 96-well microtiter plates. In each well, 5,000 B9 cells were incubated for 72 h with serial dilutions of biological samples or with different concentrations of IL-6 standard (code 89/548, National Institute for Biological Standards and Control). The number of cells in each well was measured by use of the MTT assay (see above).

Measurement of body temperature. Abdominal temperature was measured by use of battery-operated biotelemetry transmitters (VM-FH discs; Mini-Mitter, Sunriver, OR) implanted into the abdominal cavity as described previously (23–25). A Dataquest IV data acquisition system (Data Sciences, St. Paul, MN) was used for automatic control of data collection and analysis. Body temperature was monitored and recorded at 5-min intervals. For the analysis and graphical documentation, temperature data of 15-min intervals were used.

Evaluation and statistics. In graphs of the thermal responses to injections of LPS or MDP, the mean changes in abdominal temperature were plotted over time. At each time point, abdominal temperatures were expressed as means ± SE. An analysis of variance for repeated measures, followed by Scheffé’s post hoc test, was used to compare thermal responses. The calculations were carried out on an Apple Macintosh computer using the software package StatView (Abacus Concepts, Berkeley, CA). Circulating levels of TNF-α or IL-6 in response to injections of MDP or LPS were compared by Student’s t-tests or t-tests for paired observations when appropriate.

RESULTS

Experiment 1: MDP-induced production of cytokines in LPS-tolerant animals. In one group of guinea pigs, endotoxin tolerance was induced by five repeated injections of LPS in intervals of 3 days. LPS-induced production of TNF and IL-6 in response to the first, third, and fifth injection of LPS is shown on Figs. 1 and 2.

Bioactive TNF in plasma was not detectable 60 min before injection of LPS. One hour after LPS was administered, a TNF peak level of 2,610 ± 986 pg/ml was measured in response to the first of five LPS injections. This value was significantly attenuated to 933 ± 353 pg/ml after the third injection (P < 0.05) and to 123 ± 46 pg/ml after the fifth injection (P < 0.01) with LPS. Three hours after LPS administration, a mean TNF activity corresponding to 29 ± 12 pg/ml was measured in experiment 1. This value declined to 5 ± 2 pg/ml in experiment 3 and to an undetectable TNF activity in experiment 5.

One hour before injection of LPS, baseline levels of IL-6 between 15 and 70 international units (IU)/ml were measured in experiments 1, 3, and 5. Circulating IL-6 increased to 729 ± 275 IU/ml (experiment 1), 522 ± 197 IU/ml (experiment 3), and 270 ± 102 IU/ml (experiment 5) within 1 h after LPS was administered. Three hours after LPS injection, an IL-6 peak level in 1, LPS

3. LPS

5. LPS

Fig. 1. Plasma levels of tumor necrosis factor (TNF)-like activity in a group of guinea pigs (n = 7) in response to the 1st, 3rd, and 5th (1, 3, and 5, respectively) of 5 repeated intramuscular injections of 20 µg/kg LPS in intervals of 3 days. Columns represent means; bars indicate SE. n.d., not detectable; t, time.
plasma of 7,186 IU/ml was measured in response to the first injection with LPS. The corresponding IL-6 values significantly declined to 2,109 ± 797 IU/ml after the third LPS injection (P < 0.05) and to 833 ± 315 IU/ml after the fifth injection of LPS (P < 0.01). These measurements confirmed our previous report (24) that the development of LPS tolerance is associated with a corresponding attenuation of circulating TNF-α and IL-6 in guinea pigs.

Three days after the last of five LPS administrations, the LPS-tolerant guinea pigs (see Figs. 1 and 2) were injected with MDP and the production of cytokines was compared with that of a group of naive animals. The MDP-induced production of TNF and IL-6 in these two groups of guinea pigs is shown on Figs. 3 and 4.

Peak activity of TNF in response to MDP was again measured 60 min after injection of the pyrogen. The MDP-induced circulating levels of TNF at that time were identical (P = 0.483) in naive (9,924 ± 3,509 pg/ml) and in LPS-tolerant (8,910 ± 3,368 pg/ml) guinea pigs. In both groups, circulating TNF in response to MDP declined to ~300 pg/ml 3 h after MDP was administered.

One hour after MDP injection, a mean circulating bioactivity of IL-6 corresponding to 717 ± 253 IU/ml was measured in naive compared with 1,388 ± 525 IU/ml in LPS-tolerant animals. IL-6 in plasma increased within the next 2 h to 8,174 ± 2,890 IU/ml in naive and to 9,591 ± 3,916 IU/ml in LPS-tolerant guinea pigs, again statistically identical values (P = 0.66).

The results shown on Figs. 3 and 4 clearly demonstrate that LPS-tolerant guinea pigs are able to produce as much cytokines as naive animals in response to MDP.

Experiment 2: MDP-induced fever in LPS-tolerant guinea pigs. In one group of guinea pigs, endotoxin tolerance again was induced by five repeated injections of LPS in intervals of 3 days. LPS-induced fever after the first and fifth injections with LPS is shown on Fig. 5.

Compared with the first LPS-induced fever, the febrile response to the fifth injection of LPS was abro-
Fig. 6. Fever response to intramuscular injections of 100 µg/kg MDP in a group of LPS-tolerant guinea pigs (n = 7, from which the data shown on Fig. 5 have been derived) and in naive animals (n = 7). Symbols represent means; bars indicate SE.

 gated almost completely. This observation was in agreement with our previous reports on the development of a febrile tolerance to repeated LPS injections (24, 25).

Three days after the last of five LPS administrations, the LPS-tolerant guinea pigs were injected with MDP and the febrile response was compared with MDP-induced fever in naive animals. The result of this experiment is shown on Fig. 6.

MDP-induced fever in LPS-tolerant guinea pigs could not be distinguished from the febrile response to MDP in naive animals. Again, this result indicates that the development of LPS tolerance has no suppressive effect on MDP-induced fever in addition to MDP-induced production of cytokines.

**DISCUSSION**

The results of this study clearly demonstrate a lack of cross tolerance between LPS and MDP in induction of circulating TNF and IL-6 in guinea pigs. In animals that almost lost the ability to produce cytokines and to develop fever in response to LPS, the same amounts of TNF and IL-6 in plasma as in naive guinea pigs were recorded after injection of MDP. This observation provides a reasonable explanation for the reported lack of cross tolerance in the pyrogenic effects between LPS and MDP in rabbits (27) and guinea pigs (this study). The fact that animals (this study) and cells (15, 17) that lost their ability to produce cytokines in response to LPS can be triggered to an induction of TNF or IL-6 after exposure to MDP may inspire further studies on the signal transduction mechanisms leading to MDP-induced cytokine formation. According to our knowledge, the MDP-binding receptor and the MDP-induced signal pathways of cytokine induction have not yet been demonstrated or characterized.

The data shown in this (Figs. 1 and 2) and one of our previous studies (24) clearly demonstrated an association between the attenuation of fever and endogenous cytokine formation. Is the suppressed production of putative endogenous pyrogens a general phenomenon, or even the mechanism responsible for the development of tolerance to exogenous pyrogens? This question has already been addressed long ago (2). In those studies reviewed by Atkins (2), it was shown that pyrogenic tolerance developed not only to bacterial endotoxin but also to repeated injections of influenza viruses, and there was evidence that a postulated endogenous pyrogen is involved in virus-induced fever. Further data indicated that tolerance to bacterial endotoxin did not confer tolerance to influenza viruses or to the postulated endogenous pyrogen collected from plasma of febrile rabbits (2). A development of febrile tolerance to polyinosinic-polycytidylic acid was reported in guinea pigs (5), and the development of pyrogenic tolerance to MDP and a lack of cross tolerance between MDP and LPS was demonstrated in rabbits (27). The fact that rabbits and guinea pigs that have developed tolerance to one pyrogen are able to develop normal fevers in response to another pyrogen indicates that a lack of cross tolerance between distinct exogenous pyrogens may be a general phenomenon. If systemic production of cytokines (endogenous pyrogens) is necessarily involved in the generation of fever, which is questioned in current debate (4, 6, 29), it has to be postulated that unimpaired amounts of cytokines can be produced in animals tolerant to one pyrogen by stimulation with a distinct pyrogen. This has now been demonstrated for the first time for MDP-induced induction of circulating TNF and IL-6 in LPS-tolerant guinea pigs (Figs. 3 and 4). We were not able to add measurements of IL-1, the most important endogenous pyrogen, because a fever-inducing dose (in contrast to a lethal dose) of LPS is not causing large amounts of circulating IL-1 and therefore this important cytokine often escapes the limit of detection of bioassays or alternative techniques (see Ref. 13 for review).

Another question of interest is whether tolerance to MDP can be established concerning the pyrogenic and cytokine-inducing effects of this microbial product. As already mentioned, the development of febrile tolerance to MDP was demonstrated in rabbits (27). To our surprise, we recently observed that identical amounts of circulating TNF and IL-6 are released in response to each of five repeated injections of MDP in guinea pigs (23). With regard to fever, the MDP-induced elevation of nighttime temperature following the day of a first injection of this pyrogen was attenuated, whereas the initial 6–7 h of the febrile response remained manifest after each of the five repeated injections of MDP in guinea pigs (23). We are unable to explain, at present, why a significant downregulation of the cytokine network occurs in response to repeated injections of LPS but not of MDP in guinea pigs. Further studies are required to answer this question adequately.

**Perspectives**

These are the first data that indicate that the lack of pyrogenic cross tolerance between distinct exogenous pyrogens (2, 27) is associated with the ability of one pyrogen (MDP) to induce unimpaired amounts of cytokines in animals that are tolerant to another pyrogen (LPS).

The fact that tolerance to a given pyrogen (LPS) is associated with a corresponding attenuation of the
pyrogen-induced cytokine network does not explain why the production of cytokines is downregulated. A higher rate of clearance of LPS from the circulation may be responsible as well as changes of cellular elements involved in transcription or translation of cytokines.

The facts that, in response to repeated injections of MDP, unimpaired amounts of cytokines are produced in guinea pigs (23) and that LPS-tolerant guinea pigs produce as much cytokines as naive animals after injection of MDP indicate distinct receptors and signal pathways for LPS and MDP leading to cytokine formation. The characterization of the receptors activated by MDP and the subsequent intracellular signaling merits further studies at the cellular level.

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REFERENCES


