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Pyrexia, anorexia, adipsia, and depressed motor activity in rats during systemic inflammation induced by the Toll-like receptors-2 and -6 agonists MALP-2 and FSL-1

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Hübschle, Thomas, Jörg Mütze, Peter F. Mühlradt, Stefan Korte, Rüdiger Gerstberger, and Joachim Roth. Pyrexia, anorexia, adipsia, and depressed motor activity in rats during systemic inflammation induced by the Toll-like receptors-2 and -6 agonists MALP-2 and FSL-1. *Am J Physiol Regul Integr Comp Physiol* 290: R180–R187, 2006. First published September 8, 2005; doi:10.1152/ajpregu.00579.2005.—Macrophage-activating lipopeptide-2 (MALP-2) from *Mycoplasma fermentans* has been identified as a pathogen-associated molecular pattern of *Mycoplasmas* that causes activation of the innate immune system through the activation of the heterodimeric Toll-like receptors (TLRs)-2 and -6. The aim of this study was to characterize the ability of MALP-2 and a synthetic analog fibroblast-stimulating lipopeptide-1 (FSL-1; represents the NH2-terminal sequence of a lipoprotein from *M. salivarium*) to act as exogenous pyrogens, to induce formation of cytokines (endogenous pyrogens), and to cause sickness behavior, such as depressed motor activity, anorexia, and adipsia. For this purpose, body temperature, activity, food intake, and water intake were recorded for 3 days by use of telemetry devices in several groups of rats treated with MALP-2/FSL-1 or the respective control solutions. Intraperitoneal injections of FSL-1 caused fever at doses of 10 or 100 μg/kg, which was preceded by a pronounced phase of hypothermia in response to a dose of 1,000 μg/kg. The maximal fever (a peak of 1.5°C above baseline) was caused by the 100 μg/kg dose with almost identical responses to both MALP-2 and FSL-1. Fever was accompanied by pronounced rises of the proinflammatory cytokines TNF and IL-6 in plasma. Treatment with the TLR-2 and -6 agonists further induced a dose-dependent manifestation of anorexia and adipsia, as well as a reduction of motor activity. We could thus demonstrate that activation of TLR-2 and -6 can induce systemic inflammation in rats accompanied by the classical signs of brain-controlled illness responses.

fever; *Mycoplasma* infection; diacylated mycoplasmal lipopeptides; immune response; proinflammatory cytokines

The innate immune system, predominantly associated with neutrophils, monocytes, and macrophages, represents the first defense line against a variety of pathogens. Presence of a given pathogen within an infected host is recognized by so-called pathogen-associated molecular patterns (PAMPs) receptors. Members of the Toll-like receptor (TLR) family have been identified as key receptors for the recognition of PAMPs. TLR signal transduction mechanisms in myeloid, and possibly other cells, finally leads to the production of inflammatory mediators in the infected host (2, 29). LPS from gram-negative bacteria can truly be regarded as the most often studied activator of the innate immune system, acting via the TLR-4 receptor subtype of the TLR family. Before identification of TLR-4 as the crucial signal transducing receptor for LPS (27, 28), two additional accessory proteins, the LPS-binding protein and CD14 have already been described as being necessary for LPS signal transduction (for review, see Ref. 44). In vivo, the interaction of LPS with TLR-4 induces a variety of effects, including the manifestation of brain-controlled illness responses, such as fever, anorexia, or sickness behavior. At least in part, these responses are mediated by the endogenous formation of proinflammatory cytokines (7, 32).

Meanwhile, a number of distinct microbe-associated PAMPs and a number of specific TLR subtypes, recognizing those PAMPs, have been identified (2, 29). In this context, the question arose within the last few years of how *Mycoplasmas* might interact with host cells (for a review, see Ref. 34). *Mycoplasmas* form a large group of prokaryotic microorganisms that can be distinguished from ordinary bacteria by their small size and their total lack of a cell wall. The observation that *Mycoplasma fermentans* has the ability to activate macrophages in spite of the complete lack of classical bacterial PAMPs, such as LPS, lipoteichoic acid, or peptidoglycans, has led to an intensive search for a mycoplasma-specific PAMP (22). Consequently, a PAMP was identified and fully characterized biochemically as a 2-kDa lipopeptide and named macrophage-activating lipopeptide 2 (MALP-2) (23). Further studies (21, 38, 40, 41) have revealed that MALP-2 is recognized by the innate immune system through a direct activation of TLR-2 and TLR-6. First attempts to characterize the effects of MALP-2 in vivo and in vitro (12) showed that MALP-2 like LPS can be toxic to sensitized mice, that MALP-2 can induce a local skin reaction (Schwarzman reaction), and that MALP-2 can cause mitogenic activity and tumor necrosis. In the same paper (12) and in a follow-up study (8), it was reported that MALP-2 has pyrogenic activity in chinchilla and white New Zealand rabbits.

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However, a careful analysis of the pyrogenic properties of MALP-2 or MALP-2 analogs is missing.

Thus the aim of this study was to characterize MALP-2 and, because of its limited availability, also the synthetic MALP-2 analog fibroblast-stimulating lipopeptide-1 (FSL-1), as putative exogenous pyrogens in rats. We further investigated other physiological responses, such as locomotor activity and food and water intake by use of telemetry systems, because these responses are known to be modified along with LPS-induced sickness behavior (7). We finally tested the capacity of MALP-2 and FSL-1 to cause the appearance of proinflammatory cytokines in the blood. For this purpose, we measured bioactive TNF and IL-6 in blood plasma, because both of these cytokines are constantly elevated in the systemic circulation of experimental animals after systemic challenge with different PAMPs (11, 33).

MATERIAL AND METHODS

Animals

This study was performed in 118 male Wistar rats with body weights of 304 ± 4 g. Experiments were carried out in accordance with the local ethics committee (ethics approval number GI 18/2 - Nr. 59/2003). After surgery, animals were housed individually in a temperature- and humidity-controlled climate chamber (Typ 10°US/+5 to +40°DU; Weiss Umwelttechnik, Germany) at 23.5°C ambient temperature and 50% humidity. Animals had constant access to water and food and were fed with powdered standard lab chow ad libitum. With the use of special cages with water bottles and food supply dishes placed on balances (AccuScan Instruments, Columbus, OH) food and water intake was continuously monitored. Artificial lights were on from 7:00 AM to 7:00 PM. Body weight was determined once a day (~9:30 to 10:00 AM). The animals were surgically prepared for telemetric measurement of body temperature and locomotor activity 1 wk before the experiment.

Substances

To test the potential pyrogenic and anerotic effects of agonists that lead to TLR-2 and -6 activation in rats, two distinct TLR-2 and -6 agonists were used in this in vivo study, e.g., the diacylated macrophage-activating lipopeptide from M. fermentans (MALP-2, Pam2Cys-GNNDENISNFKEK) and the diacylated FSL-1 (Pam2Cys-GDPKHPKSFP), representing the NH2-terminal sequence of the 44-kDa lipoprotein LP44 of M. salivarium (24, 25, 39). MALP-2 was synthesized and purified by high-pressure liquid chromatography as previously described (21, 23). MALP-2, stored as a sterile, lyophilized, endotoxin-free powder, was diluted in 10% Cremophor EL-90% sterile PBS. Cremophor EL is a nonionic solubilizer and emulsifier designed for in vivo use in human and veterinary pharmaceuticals. Cremophor EL was a kind gift from BASF Aktiengesellschaft (Ludwigshafen, Germany). MALP-2 was used in a dose of 100 μg/kg body wt. Control experiments were performed with 10% Cremophor EL in 90% PBS (solvent). Synthetic FSL-1 was purchased as a mixture of the RR and RS stereoisomers (prod. no. L7000; EMC microcollections, Tu¨bingen, Germany). According to EMC microcollections, test the RR and RS stereoisomers (prod. no. L7000; EMC microcollections, Tu¨bingen, Germany). According to EMC microcollections, synthetic FSL-1 was used in three different doses of 10, 100, and 1,000 μg/kg body wt. Control experiments were performed with pyrogen-free PBS only.

Measurement of Body Temperature and Locomotor Activity

Abdominal temperature was measured in 58 male Wistar rats by using biotelemetry transmitters (VM-FH-discs; Mini-Mitter) implanted 1 wk before the experiment into the abdominal cavity. During the surgery, rats were anesthetized with 100 mg/kg ketamine hydrochloride (Albrecht, Aulendorf, Germany) and 4 mg/kg xylazine (Bayer Vital, Leverkusen, Germany). The output (frequency in Hz) was monitored by a receiver placed under each cage (model RA 1000; Mini-Mitter). A data acquisition system (Vital View; Mini-Mitter) was used for automatic control of data collection and analysis. Body temperature was continuously recorded at 5-min intervals. For analysis and graphical documentation, temperature data at time intervals of 5 min (Fig. 3), as well as 15 min (Fig. 1), were used. Locomotor activity of rats was measured using the same biotelemetry system described above. Changes in activity were detected by changes in the position of the implanted transmitter over the receiver board. This resulted in a change of the signal strength detected by the receiver and recorded as a pulse of activity. Activity pulses were counted every 5 min and were either documented as a continuous histogram (counts per 5 min, Fig. 3) or added for 12 h as a cumulative measure of daytime or nighttime activity and expressed as activity counts per 12 h (Fig. 4).

Cytokine Bioassays

Cytokine analysis was performed with plasma samples from 60 male Wistar rats stimulated either with TLR-2 and -6 agonists or the respective control vehicles. Determination of TNF was achieved by a bioassay based on the cytotoxic effect of TNF on the mouse fibrosarcoma cell line WEHI 164 subclone 13 (10). The assay was performed in sterile, 96-well microtiter plates. Serial dilutions of biological samples or different concentrations of a murine TNF-standard (code 38/532, National Institute for Biological Standards and Control, South Mimms, UK) were incubated for 24 h in wells seeded with 50,000 actinomycin D-treated WEHI cells. The number of surviving cells after 24 h was measured by using the dimethylthiazol-diphenyl tetrazolium bromide (MTT) colorimetric assay (13). Plasma samples were prediluted so that serial dilution of samples and standard dilution curves were parallel. The detection limit of the assay, after considering the dilution of samples into the assays, was 6 pg/ml TNF.

Determination of IL-6 was achieved by a bioassay based on the dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line (1). 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 a human IL-6 standard (code 89/548, National Institute for Biological Standards and Control). Plasma samples were prediluted so that serial dilution of samples and standard dilution curves were parallel. The number of cells in each well was measured by use of the MTT assay (see above). The detection limit of the assay, after considering the dilution of samples into the assays, was 3 IU of IL-6/ml.

As opposed to ELISA assays, use of the described bioassays does not exclude the possibility that some undiscovered substance might interfere in the assay by causing proliferation of the B9 cells or cytotoxicity to the WEHI cells. At least for the TNF assay, we are able to exclude this possibility because a small amount of a synthetic form of the soluble type 1 TNF receptor (33) completely neutralized TNF bioactivity in some tested plasma samples. A similar test for the IL-6 assay was not available for our study. Therefore, we would have to refer to the measured IL-6 as IL-6-like-activity. We further tested possible interference of FSL-1 or MALP-2 in our assay systems. Below concentrations of 5 μg/ml, both FSL-1 and MALP-2 had no effects on B9 or WEHI cells. At this concentration, which could not even theoretically appear in our plasma samples, MALP-2, but not FSL-1, had a measurable cytotoxic effect on both cell lines.

Measurement of Food and Water Intake

Food intake and drinking behavior were telemetrically monitored at 5-min intervals by using special cages equipped with water bottles and food supply dishes placed on balances, which in turn, were connected
via a DietScan analyzer to a personal computer (AccuScan Instruments). The AccuDiet software package (AccuScan Instruments) was used to record and later, to transfer the data for graphical and statistical analysis. Cumulative food and water intake per 5 min were combined into cumulative measure of daytime or nighttime food and water intake over a 12-h period. Therefore, the final data represent cumulative food and water intake in grams per 12 h (Fig. 4).

### Experimental Protocols

**Experiment 1.** Six groups of rats were injected intraperitoneally between 9:30 and 10:00 AM with a total injection volume of 1 ml each. Three different doses of FSL-1 (10 μg/kg, n = 5; 100 μg/kg, n = 12; 1,000 μg/kg, n = 4) or an equivalent volume of the vehicle (PBS, n = 13) were injected. Another two groups of rats received intraperitoneal injections of 100 μg/kg MALP-2 (n = 13) or an equivalent volume of the solvent for this drug (n = 11). For all of these six groups of rats, body temperature was evaluated from 2 h before until 9 h after the intraperitoneal stimulation. This time interval was chosen because it ended before the start of the “lights-off” period. The 15-min intervals values were corrected to the actual time point of injection.

**Experiment 2.** Male Wistar rats were injected intraperitoneally with the TLR-2 and -6 agonists FSL-1 or MALP-2 at a dose of 100 μg/kg (n = 4 per time point) or the respective vehicle solutions (n = 3 per time point) to study the endogenous formation of cytokines in response to TLR-2 and -6 activation. Blood was collected at 60, 120, 180, 240, or 420 min after the time of injection. Because of the limited availability of MALP-2, blood was collected only at 60, 120, or 240 min after the injection of MALP-2 or its vehicle. The basal values of both cytokines were determined in four animals at a time point corresponding to a time point 1 h before intraperitoneal stimulation, e.g., −60 min. Plasma samples were collected by cardiac puncture. Briefly, 5 min before the scheduled time of blood collection, the rats were deeply anesthetized with 60–100 mg/kg pentobarbital sodium (Narcoren; Merial, Hallbergmoos, Germany) injected intraperitoneally, and 5 min later, blood was withdrawn into sterile heparinized syringes by cardiac puncture. After centrifugation, plasma was stored at −70°C for later determination of bioactive TNF and IL-6.

**Experiment 3.** A detailed analysis of the physiological parameters, i.e., body temperature, motor activity, and food and water intake, as well as changes in body weight, were performed within the 3 days following intraperitoneal stimulation with the TLR-2 and -6 agonists or their respective control solutions. The analysis starts with the beginning of the lights-on period on day 1, e.g., about 2–3 h before stimulation. This long-term analysis was done with a subset of animals already described for experiment 1. Only data of those animals were included in this analysis, of which the complete set of data was available for all physiological parameters. In detail, six groups of rats were injected intraperitoneally between 9:30 and 10:00 AM with a total injection volume of 1 ml each. Three different doses of FSL-1 (10 μg/kg, n = 5; 100 μg/kg, n = 7; 1,000 μg/kg, n = 4) or an equivalent volume of the vehicle (PBS, n = 7) were injected. Another two groups of rats received intraperitoneal injections of 100 μg/kg MALP-2 (n = 9) or an equivalent volume of the solvent for this drug (n = 8).

### Evaluation and Statistics

All data are given as means ± SE. Statistical calculations were carried out with the SigmaPlot/SigmaStat analysis software (SPSS Science Software, Erkrath, Germany). Concentrations of bioactive cytokines in blood and cumulative data on locomotor activity, food and water intake, and body weight changes were compared by one-way ANOVA followed by an appropriate post hoc test. Abdominal temperatures were compared between different treatment groups over time by two-way repeated-measures ANOVA followed by an all-pairwise Bonferroni’s multiple comparison post hoc test. Statistical significance was accepted at P < 0.05.

### RESULTS

#### Experiment 1: Fever in Response to Systemic Treatment with the TLR-2 and -6 Agonists FSL-1 and MALP-2

Intraperitoneal injections of 10, 100, or 1,000 μg/kg FSL-1 induced distinct thermal responses in rats as shown in Fig. 1. **Top.** All groups of rats, including the control group injected with PBS, exhibited a transient hyperthermia lasting about 60 min with a peak at 30 min after administration of the respective solution. This rise of abdominal body temperature (T_{abd}) was most likely caused by stress during the injection procedure. In rats treated with a dose of 10 μg/kg FSL-1 or with PBS, abdominal temperature returned to its baseline within 60 min. Control animals showed no further fluctuations of T_{abd}, whereas rats injected with the lowest of the tested FSL-1 doses developed a fever that started 165 min after injection and lasted until the end of the lights-on period. In response to injections of 100 μg/kg FSL-1, rats developed a slight drop of T_{abd} immediately after the stress-induced hyperthermia, followed by a fairly strong fever with a duration that even exceeded the lights-on period of the day of FSL-1 administration. The highest dose of FSL-1 (1 mg/kg) induced a pronounced hypo-
thermia between 60 and 225 min after injection. Thereafter, $T_{\text{ab}}$ increased above the baseline values so that a fever developed from 225 until 480 min after injection of 1 mg/kg FSL-1. For reasons of clarity, only those time points or time intervals are shown with significance bars in Fig. 1, top, at which all of the three tested doses of FSL-1 caused a significant elevation of $T_{\text{ab}}$ compared with rats treated with PBS ($P < 0.05$).

To compare the pyrogenic capacity of MALP-2 to that of its synthetic analog FSL-1, a dose of 100 μg/kg was used, because this dose of FSL-1 had the most pronounced pyrogenic effect (Fig. 1, top). As shown in Fig. 1, bottom, intraperitoneal injections of 100 μg/kg MALP-2 and FSL-1 caused thermal responses of almost identical shape and duration. Stress-induced hyperthermia was followed by a transient drop of $T_{\text{ab}}$ and a pronounced fever that seemed to be slightly higher in MALP-2-treated rats. The significance bars and stars indicate only those time points or time intervals at which both pyrogens FSL-1 and MALP-2 caused significant increases or decreases of $T_{\text{ab}}$ compared with their respective control groups injected either with PBS or with solvent ($P < 0.05$).

**Experiment 2: Circulating Cytokines in Response to Systemic Treatment with the TLR-2 and -6 Agonists FSL-1 and MALP-2**

Levels of bioactive IL-6 in plasma at different intervals from the time of injection of 100 μg/kg FSL-1 compared with PBS or of 100 μg/kg MALP-2 compared with solvent are shown in Fig. 2, top.

The baseline activity of IL-6 in plasma (80 IU/ml, measured 60 min before the respective injections) was not altered significantly by injections of PBS or solvent. Injections of FSL-1 or MALP-2, however, caused drastic increases in the plasma levels of IL-6. In FSL-1-treated rats, IL-6 in plasma showed a 12-fold increase already 1 h after injection compared with the respective control value. There was a further increase to 32-fold (2 h), 60-fold (3 h), and even 95-fold (4 h) higher levels of IL-6 in plasma within the following hour after injection of FSL-1. At 420 min after injection, FSL-1-induced circulating IL-6 already declined to a mean value that was still 17-fold higher than in control rats. At all investigated time intervals, IL-6 in plasma of FSL-1-treated rats was significantly elevated compared with controls ($P < 0.05$). Kinetics of MALP-2-induced bioactive IL-6 in plasma was similar, but a bit less pronounced when compared with the data of FSL-1-injected rats. There was a ninefold rise within the first hour after MALP-2 treatment, which further increased to 23-fold (2 h) and 62-fold (4 h) higher levels compared with the respective control values. At each investigated time point, the MALP-2-induced circulating amounts of bioactive IL-6 were significantly higher than in animals injected with solvent ($P < 0.05$).

Bioactive TNF was not detectable in plasma 60 min before any of the respective injections and remained undetectable in PBS- or solvent-treated rats at all investigated time points after administration of both vehicles. Intraperitoneal injection of 100 μg/kg FSL-1 caused a quick increase in circulating TNF within 1 h to a mean level of 5,500 pg/ml. This value further increased to 6,080 pg/ml at 2 h, and rapidly declined to 125 pg/ml at 3 h after FSL-1-treatment. At later time points, plasma TNF was below the detection limit of the TNF-bioassay. MALP-2-induced mean circulating concentrations of bioactive TNF were 1,820 pg/ml at 1 h and 4,880 pg/ml at 2 h after injection. Taken together, both FSL-1 and MALP-2 at a dose of 100 μg/kg injected intraperitoneally caused the appearance of pronounced amounts of bioactive IL-6 and TNF in the blood. Levels of FSL-1-induced cytokines were slightly higher compared with the responses of MALP-2-treated rats at the corresponding time points.

**Fig. 2. Circulating proinflammatory cytokines in rats injected intraperitoneally with FSL-1, MALP-2, or vehicle. Time course of plasma IL-6 (top), and TNF (bottom) concentrations in response to 100 μg/kg FSL-1 (left) and MALP-2 (right) or to the respective vehicles (PBS or solvent). Asterisks indicate significant differences between TLR-2 and -6 agonist-treated rats and their respective controls ($P < 0.05$). Plasma concentrations of bioactive TNF proved to be under the lower detection limit (ud) in all samples collected from control animals and at time intervals later than 180 min after injection of the respective TLR-2 and -6 agonists.**
Experiment 3: Three-Day Analysis of Physiological Responses to Systemic Treatment with the TLR-2- and -6 Agonists FSL-1 and MALP-2

Results of an analysis of various physiological responses to FSL-1 or MALP-2, all recorded by telemetric devices, are shown in Figs. 3 and 4. In Fig. 3, the continuous recordings of T_{abdominal} and motor activity for a period of 3 days starting with the lights-on period of the day of the injection of 100 μg/kg FSL-1, or of 100 μg/kg MALP-2, or of the respective vehicles are documented. In the first night after the day of injection (night 1, Fig. 3A) T_{abdominal} of FSL-1- or MALP-2-treated rats did not continue to increase and finally returned to the normal nighttime values of control animals. There was a tendency for slightly elevated core temperatures of pyrogen-treated rats during day 2 and day 3, whereas the night-time temperatures were identical to those of the control groups. Generally, the circadian temperature rhythm of rats injected with FSL-1 or MALP-2 was not disturbed during the second and third days and nights. Motor activity was depressed predominantly in the first night after the injection of FSL-1 or MALP-2 (Fig. 3, B and D). The detailed statistical analysis of motor activity, cumulative water or food intake, and development of body weight in all investigated animal groups is summarized in Fig. 4.

Fig. 4A shows the mean cumulative motor activities separated for 3 days and 3 nights starting with the day of the respective injection. Treatment with FSL-1 significantly re-
duced motor activity during night 1 at doses of 100 μg/kg or 1,000 μg/kg, whereas the lowest dose (10 μg/kg FSL-1) had no significant influence on the animals’ activity. MALP-2, at a dose of 100 μg/kg, had a stronger and longer-lasting impact on the rats’ motor activity compared with FSL-1 at the same dose. In response to MALP-2, the mean number of activity counts was significantly reduced during all of the three following nighttime periods compared with control rats injected with solvent. Even the generally lower daytime activity was significantly reduced on the day of the injection of MALP-2.

Cumulative water intake was significantly reduced during night 1 in rats injected with 100 or 1,000 μg/kg FSL-1 and in response to injection of MALP-2 at a dose of 100 μg/kg (Fig. 4B). Only the highest dose of FSL-1 had a depressive effect on water intake during night 2. In the third night after the respective injections, cumulative water intake normalized in all groups treated with the TLR-2 and -6 agonists. The effect of treatment with FSL-1 or MALP-2 on cumulative food intake (Fig. 4C) was more pronounced compared with the adipisa caused by both of the tested putative exogenous pyrogens. There was a significant anorectic effect of the highest dose of FSL-1, which lasted at least for three nights. At a dose of 100 μg/kg, FSL-1 significantly reduced food intake during the first two nights following the day of injection, while the same dose of MALP-2 caused a significant reduction of food intake during night 1 and night 3 and a tendency for depression of food intake during night 2. In response to the smallest of the tested doses of FSL-1 (10 μg/kg), neither adipisa nor anorexia developed.

In line with the depression of food intake, rats treated with 100 μg/kg, 1,000 μg/kg FSL-1, or 10 μg/kg MALP-2 developed a significant reduction of body weight, which did not yet normalize during the first 3 days after injection compared with the respective control groups (Fig. 4D).

**DISCUSSION**

**Activation of the Innate Immune System by MALP-2/FSL-1**

Mammalian TLRs play a crucial role as sensors of infection and induce a rapid activation of the innate immune response (3, 14). For the signal transduction induced by LPS from gram-negative bacteria via TLR-4, first a transport by LPS-binding protein and then recognition by CD14, the coreceptor on the surface of macrophages and other cell types, is required (44). Interaction of TLR-4 with the complex formed by LPS, its binding protein, and CD14 causes transcriptional activation of target cells and induces activation of the transcription factors NF-kB, AP1, and others (for a review, see Ref. 18). Thereby, LPS- and TLR-4-dependent transcription of target genes, including those for proinflammatory cytokines is controlled (2). Likewise, the TLR-2- and -6-mediated activation of intracellular signal transduction pathways is finally also leading to a pronounced formation of the proinflammatory cytokines TNF and IL-6 in vitro (8, 40, 41; for a review, see Ref. 4) and in vivo (8) (Fig. 2). Both cytokines are regarded as endogenous mediators of brain-controlled signs of illness (6, 7, 20, 26, 32). Thus the central goal of this study was to analyze the manifestation of fever, motor activity, anorexia, and adipisa in rats in response to MALP-2 and FSL-1 in relation to a putative appearance of measurable amounts of cytokines in the blood.

**Brain-Controlled Illness Responses Induced by MALP-2 and FSL-1**

In a first experiment, we determined a dose of FSL-1, which produced pronounced fever. The febrile responses of rats to 10, 100, or 1,000 μg/kg FSL-1 showed some similarities to LPS-induced changes of T<sub>ab</sub>. It has been reported (31) that fever develops in rats in response to 10 or 100 μg/kg LPS, whereas a dose of 1,000 μg/kg LPS causes hypothermia for several hours. A similar response pattern was observed in our experiments. Doses of 10 and especially of 100 μg/kg FSL-1 induced fever that was preceded by a pronounced phase of hypothermia in response to the 1,000 μg/kg FSL-1 dose. In response to higher doses of LPS and also of FSL-1 as shown in Fig. 1, it seems that systemic inflammation frequently consists of phases of regulated hypothermia and fever, both of which may be of adaptive value (17). At a dose of 100 μg/kg, both FSL-1 and MALP-2 induced pronounced and almost identical fevers that were preceded by a short phase of hypothermia. It has to be noted that all fever experiments were made at a subneutral ambient temperature, which might have modified the early phase of the thermal responses to diacylated lipopeptides as reported for LPS-induced febrile and hypothermic phases (30, 35).

Fever induced by FSL-1 and MALP-2 was accompanied by a substantial rise of TNF and IL-6 in plasma. Increase of circulating TNF predominantly occurred during the period when we observed a moderate drop of T<sub>ab</sub>. Elevation of circulating IL-6 seemed to persist until the fever fully developed. Increased levels of circulating cytokines in response to FSL-1 and MALP-2 were thus not preceded by the onset of fever, as has frequently been reported for LPS (for a review, see Ref. 5). Thus it seems plausible that the cytokines released into the bloodstream after injection of FSL-1 or MALP-2 contributed to the observed changes of T<sub>ab</sub> (for a review, see Ref. 9).

With the exception of the lowest dose of FSL-1 (10 μg/kg), all treatments with TLR-2 and -6 agonists induced significant depression of motor activity, anorexia, and adipisa. These components of sickness behavior are also caused by comparable treatments with LPS or other bacterial and viral pyrogens and their respective PAMPs (11, 19, 37, 42, 45). All of these responses, especially the depression of food intake and the concomitant depression of body weight after the administration of FSL-1 or MALP-2, lasted longer than the observed fever and probably also longer than the elevations of proinflammatory cytokines in the blood. It has been suggested that a secondary production of brain-intrinsic cytokines and a subsequent activation of brain-intrinsic cytokine receptors triggers those long-lasting behavioral effects, which follow the treatment with various bacterial or viral pyrogens (7, 11, 26, 37). However, at least at the beginning of the inflammatory process, the activation of TLRs seems to be a prerequisite for the manifestation of brain-controlled illness responses. Studies in mice genetically deficient in CD14, TLR-4, or TLR-2 revealed that the anorectic effect of LPS was blunted in TLR-4- or CD14-deficient mice; in contrast, anorexia induced by treatment with muramyl-di-pentapeptide (an activator of TLR-2 from gram-positive bacteria) was attenuated in TLR-2 knockout mice (45). In that context, it is important to note that the signal transduction pathway of muramyl-di-pentapeptide is controversially...
discussed, possibly due to undetected impurities in some muramyl-dipeptide preparations. Indeed, other studies conclude that muramyl-dipeptide is recognized by an internal cell receptor of the nucleotide-binding oligomerization domain protein family (15, 43), thereby acting via a different signaling mechanism totally independent fromTLRs. Comparable experimental studies on fever in such mice are still missing, but it can be expected that the lack of a particular TLR will have an impact on the febrile response induced by the respective TLR agonist.

Perspectives for Future Studies

For more than half a century, LPS has been regarded as almost unique in mediating many (patho)physiological activities. Shortly after the discovery and characterization of MALP-2 from M. fermentans (21, 23), it became obvious that this substance has classic endotoxic properties (12). Such a view is supported by our study with special regard to the ability of MALP-2 and FSL-1 as exogenous pyrogens, stimulator of cytokine formation in vivo, and inducer of sickness behavior. Here the question arises, if there is a constitutive or stimulus-dependent upregulated expression of TLR-2 and -6 in the brain, which may account for the observed brain-intrinsic cytokine formation after administration of Mycoplasmas directly into the brain. Alternatively, TLR-2 and -6 activation might possibly be induced at the interface between the blood and the brain, i.e., at the level of the so-called sensory circumventricular organs lacking a tight blood-brain barrier. In some of these specialized brain structures, CD14 and TLR-4, the receptive molecules for LPS, have been demonstrated (16). A further question of interest concerns the investigation of fever and other components of the acute-phase response in TLR-2- and TLR-6 deficient mice. Finally, it seems worthwhile to investigate whether TLR-2 and -6 stimulation by MALP-2 and FSL-1 will cause similar transcriptional brain activation as has been documented for LPS via its endogenous mediators (36). Answers to these questions would provide additional arguments for the postulation of the “end of an era of LPS monopoly” after the discovery and characterization of MALP-2 (12).

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REFERENCES


