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Induction of Fever and Sickness Behavior in Telemetrically Monitored Rats During Systemic Inflammation Induced by Zymosan

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Abstract: Zymosan (Zy) has been shown to act as a pathogen-associated molecular pattern that activates the innate immune Toll-like receptor subtypes 2 and 6 (TLR2, TLR6). Systemic challenge with TLR2/6-agonists has been shown to mediate brain-controlled signs of sickness behavior amongst them fever, depressed motor activity, anorexia and adipsia. This study was designed to determine whether Zy as a further TLR2/6-agonist is also capable to induce similar sickness behavior responses during systemic Zy-induced inflammation as those observed with Mycoplasma-specific TLR2/6 agonists. In telemetrically monitored rats intraperitoneal treatment with 10 mg kg^{-1} Zy induced a moderate febrile response with significantly elevated abdominal temperatures (ΔT of about 1.2°C) for a period of about 4 h on the day of injection (day 1). Already at the end of day 1 temperature data of the Zy group returned to normal values of control animals and thereafter showed an almost identical circadian rhythm for the next 3 nights and 2 days. Zy-treated animals showed generally lower cumulative motor activities, however, significantly reduced only during night 1 and night 3. Cumulative food intake was significantly reduced during night 1 and night 2 in the Zy group, while no significant changes were measured for cumulative water intake. In line with the depressed food intake, Zy-treated rats developed a reduction in body weight gain that became significant on day 3 and day 4 after the injection. In conclusion, Zy-induced fever, anorexia and depressed motor activity seem to result from activation of cellular pathways involved in the TLR2/6-dependent innate immune responses.

Key words: Anorexia, body temperature, locomotor activity, telemetry, toll-like receptor 2 and 6, immune response

INTRODUCTION

Within the last years strong research efforts have been directed to the identification and characterization of the recognition system of the innate immune system for various Pathogen-Associated Molecular Patterns (PAMPs). Toll-Like Receptors (TLRs) represent an initial and important component of this recognition system (Aderem and Ulevich, 2000) and TLR2 is one important mammalian receptor activated by various PAMPs, including the Mycoplasma-specific lipopeptides Macrophage-Activating Lipope Ptide-2 (MALP-2) and its analogue fibroblast-stimulating lipopeptide-1, peptidoglycans from gram-positive bacteria as well as the nonbacterial agent Zymosan (Zy) (Dziarski and Gupta, 2005; Takeuchi *et al*., 1999; Takeda *et al*., 2003; Muhlradt and Frisch, 1994; Muhlradt *et al*., 1997; Underhill *et al*., 1999). Zy is a prominent polysaccharide cell wall component from the yeast *Saccharomyces cerevisiae*. Its TLR2-mediated activation of the innate immune system can be mediated by the transcription factor Nuclear Factor kappa B (NFkB) and leads to the transcriptional activation of various immune response genes such as the proinflammatory cytokine Tumor Necrosis Factor (TNF), the interleukins 1 and 6 (IL-1, IL-6) (Sato *et al*., 2003; Bondeson *et al*., 1999). Once produced and released from immune competent cells these proinflammatory cytokines in turn seem to play an important role in the induction and maintenance of brain-controlled signs of illness commonly referred to as sickness behavior (Dantzer, 2004). Interestingly, experiments in rats systemically inflamed by *in vivo* treatment with two other TLR2/6 agonists, MALP-2 and FSL-1, have suggested a TLR2/6-mediated induction of brain-controlled signs of sickness behavior amongst them fever and depressed motor activity, anorexia and adipsia (Hubschle *et al*., 2006). Not surprisingly, those responses were accompanied by pronounced rises of TNF and IL-6 in plasma on the day of injection.

Treatment with Zy in rodents is used as animal models to induce systemic inflammation and depending on the Zy dose, even used to create a septic shock-like

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response and multiple organ damage and dysfunction (Volman *et al*., 2005). The influence of Zy on behavior has received little attention. One previous study has already reported that Zy elicited sickness behavior in a dose-responsive manner in female rats (Cremeans *et al*., 2003). However, this study did not use up to date telemetric devices to monitor the behaviors elicited by the Zy treatment and for example body temperature was determined with rectal temperature probes. Our study was designed to determine whether Zy as one further TLR2/6-activating PAMP, is capable to induce similar sickness behavior responses during systemic Zy-induced nonseptic inflammation as those observed with the Mycoplasma-specific TLR2/6 agonists *in vivo* (Hubschle *et al*., 2006). Continuous telemetric measurement of body temperature, motor activity as well as food and fluid intake was used to assess potential Zy effects on these behaviors in male Wistar rats.

MATERIALS AND METHODS

Animals: The study was performed in 24 male Wistar rats with body weights of 275±4.5 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 (Weiss Umwelttechnik GmbH, Typ 10'US/+5 - +40 DU, Germany) at 23.5°C ambient temperature and 50% humidity. Animals had constant access to water and were fed with powdered standard lab chow *ad libitum*. By use of special cages with water bottles and food supply dishes placed on balances (AccuScan Instruments, Columbus, USA) food and water intake was continuously monitored. Artificial lights were on from 7:00 AM to 7:00 PM. Body weight was determined once on a day (~9:30 to 10:00 AM). The animals were surgically prepared for telemetric measurement of body temperature and locomotor activity one week before the experiment.

Substances: Zymosan A from Saccharomyces cerevisiae (Sigma, Taufkirchen, Germany) was suspended in sterile Phosphate-Buffered Saline (PBS) and stored at -20°C as a stock solution at 10 mg mL^{-1}. Just prior to injection Zy stocks were suspended and agitated in PBS at 10 mg kg^{-1} body weight and injected in a total volume of 1 mL. This dose was chosen from own pilot experiments in which no obvious Zy-induced hypothermic effect was observed in rats. None of our animals died from Zy treatment. Control experiments were performed with pyrogen-free PBS only.

Measurement of body temperature and locomotor activity: Abdominal temperature was measured in rats by use of biotelemetry transmitters (VM-FH-discs, Mini-Mitter, Bend, Oregon, USA) implanted 1 week before theexperiment into the abdominal cavity. During the surgery rats were anaesthetized with 100 mg kg^{-1} ketamine hydrochloride (Albrecht, Aulendorf, Germany) and 4 mg kg^{-1} xylazin (Bayer Vital, Leverkusen, Germany). The output (frequency in Hz) was monitored by a receiver placed under each cage (RA 1000, Mini-Mitter, Bend, Oregon, USA). A data acquisition system (Vital View, Mini-Mitter, Bend, Oregon, USA) 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 as well as 15 min (Fig. 1) were used. The locomotor activity of rats was measured using the same biotelemetry system. 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, which was detected by the receiver and recorded as a pulse of activity. Activity pulses were counted every 5 min and either documented as a continuous histogram (counts per 5 min, Fig. 2), or added for 12 h as a cumulative measure of day-time or night-time activity and expressed as activity counts per 12 h (Fig. 3).

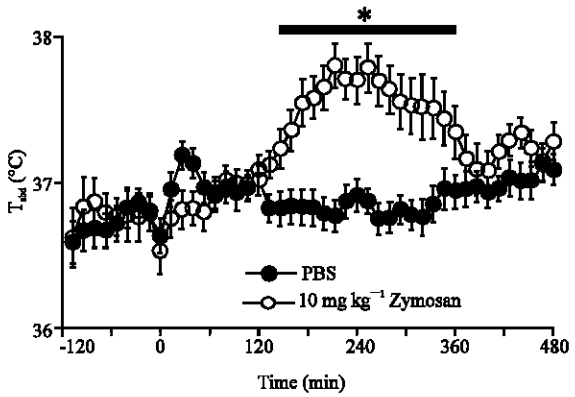

Fig. 1: Febrile responses of rats injected intraperitoneally with zymosan or vehicle Rats were injected with 10 mg kg^{-1} zymosan (N = 12, open circles) or vehicle (N = 12, black circles) at time point 0. At those time points indicated by significance bar and asterisk a significant zymosan-induced elevation of abdominal body Temperature (T_{abd}) compared with rats treated with sterile PBS was observed ($p<0.05$)

Measurement of food and water intake: Food intake and drinking behavior were telemetrically monitored at 5 min intervals by use of special cages equipped with water bottles and food supply dishes placed on balances, which in turn were connected via a Diet Scan analyser to a

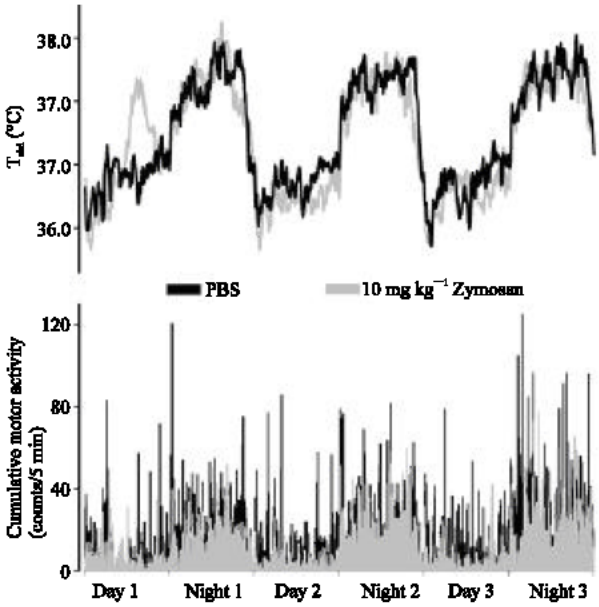

Fig. 2: Circadian rhythm of body temperature and motor activity in rats injected intraperitoneally with zymosan or vehicle Long term and high resolution documentation of mean values of abdominal body Temperature (T_{abd}, upper panel) and cumulative motor activity (lower panel) for 3 days and nights. The measurement starts with the day of injection in rats treated with 10 mg kg^{-1} zymosan (N = 9, grey curve and bars) or the vehicle sterile PBS (N = 6, black curve or bars)

personal computer (AccuScan Instruments, Columbus, USA). The AccuDiet software package (AccuScan Instruments, Columbus, USA) 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 day-time or night-time 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. 3).

Experimental protocols

Experiment 1: Two groups of rats were injected i.p. between 9:30 and 10:00 AM with a total injection volume of 1 mL each. Zy was administered intraperitoneally in a nonseptic and nonhypothermic dose of 10 mg kg^{-1} body weight (N=12). In control animals an equivalent volume of the vehicle (PBS, N = 12) was injected. Body temperature was evaluated from two hours before until 9 h after the i.p. stimulation. This time interval was chosen because it ended prior to the start of the light-off period. The 15 min intervals values were corrected to the actual time point of injection.

Experiment 2: A detailed analysis of the physiological parameters, i.e., body temperature, motor activity, food and water intake as well as changes in body weight was performed by use of biotelemetry within the three days following i.p. stimulation with Zy or PBS. The analysis starts with the beginning of the light-on period on day 1, e.g., about 2-3 h prior to stimulation. This long term analysis was done with a subset of animals already described for experiment 1. Only data of those animals were included into this analysis of which the complete set of data was available for all physiological parameters. Rats were injected i.p. between 9:30 and 10:00 AM with a total injection volume of 1 mL with either Zy (N = 9, 10 mg kg^{-1}) or PBS (N = 6).

Evaluation and statistics: All data are given as means±SEM. Statistical calculations were carried out with the Sigma Plot Sigma Stat analysis software (SPSS Science Software GmbH, Erkrath, Germany). Abdominal temperatures, cumulative data on locomotor activity, food and water intake and body weight changes were compared between the two different groups over time by two-way repeated-measures ANOVA followed by an all pairwise Bonferroni's multiple comparison *post hoc* test. A statistical significance was accepted for $p<0.05$.

RESULTS

Experiment 1: Moderate fever in response to systemic treatment with 10 mg kg^{-1} zymosan: When compared to the normal abdominal Temperature (T_{abd}) data of control animals that received sterile PBS, i.p. injections of 10 mg kg^{-1} Zy induced a significant elevation (ΔT of about 1.2°C) of T_{abd} for a period of about 4 h starting 165 min after its injection. Already 7 h after zymosan injection T_{abd} dropped again and did not significantly differ any more from the temperature values observed in the control group. Note that the initial rise of T_{abd} in the control group 30-60 min after the injection was most likely caused by stress during the injection procedure. This stress-induced rise in body temperature was much less pronounced in Zy-treated rats. Still the statistical analysis showed no significant difference between both groups. Fig. 1.

Experiment 2: Three-day analysis of physiological responses to systemic treatment with 10 mg kg^{-1} zymosan: The results of an analysis of various physiological responses to 10 mg kg^{-1} Zy, all recorded by telemetric devices, are shown in Fig. 2 and 3. In Fig. 2, the continuous recordings of T_{abd} and motor activity mean

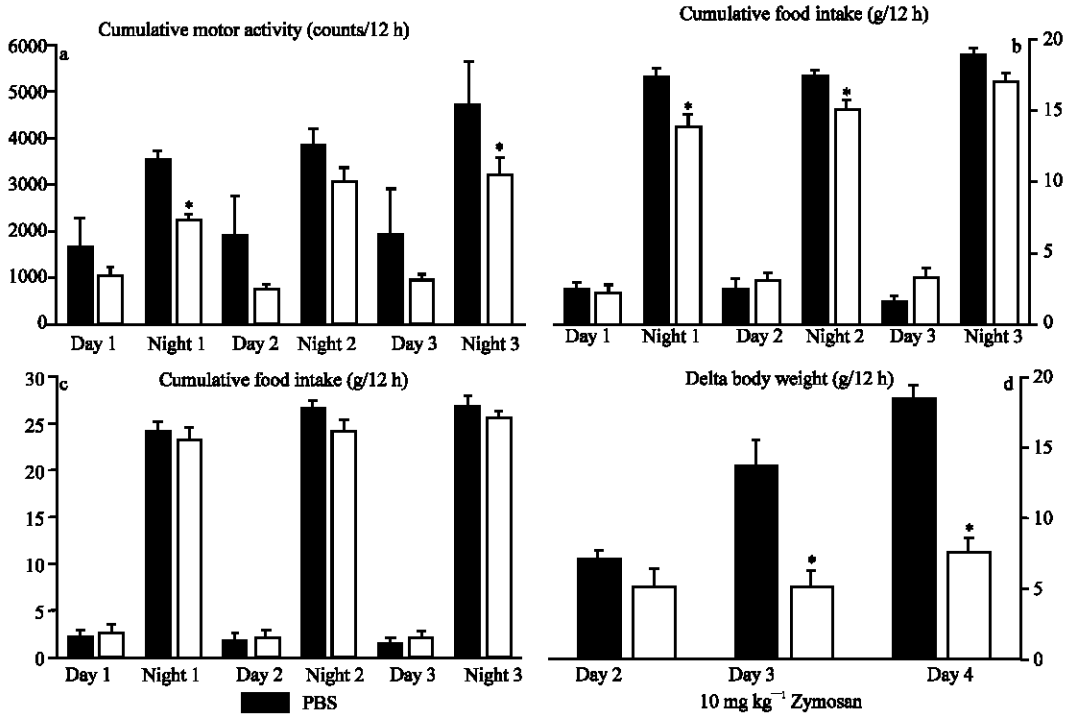

Fig. 3: Changes in cumulative 12 or 24 h values of motor activity, food and water intake as well as body weight in rats injected intraperitoneally with zymosan or vehicle Cumulative motor activty (a), food intake (b), water intake (c)and body weight (d) are shown for rats treated with 10 mg kg^{-1} zymosan (N = 9, open bars) or the vehicle sterile PBS (N = 6, black bars). Mean cumulative motor activity, food and water intake were calculated for each lights-on and lights-off period (days 1-3, nights 1-3), whereas body weight was determined on a daily basis. Asterisks indicate a significant zymosan-induced change of either parameter when compared with rats treated with sterile PBS ($p<0.05$)

values are shown for both groups for a period of 3 days starting with the lights-on period on the day of injection. This analysis clearly revealed the rise in T_{abd} in Zy-treated rats during the day of injection (day 1) as already observed for the short term analysis in Fig. 1. With the end of day 1 temperature data of the Zy group returned to normal values of control animals and thereafter showed an almost identical circadian rhythm for the next 3 lights-off (nights 1-3) and 2 lights-on periods (days 2-3) (Fig. 2).

As for the continuous recording of cumulative motor activity over the three-days period no such clear difference could be observed between both groups like that seen on day 1 for T_{abd}. The general activity pattern seemed to be identical with less activity during the day and more at night. However, a tendency of lower day as well as night activities was observed in the Zy-treated group when compared to the control group (Fig. 2. lower panel). Then again the detailed statistical analysis of cumulative motor activities during the whole day and night periods, revealed that although there exists a tendency of generally lower activities they differ significantly only in night 1 and night 3 from each other (Fig. 3a).

Figure 3b-d shows the detailed statistical analysis of the other physiological parameters, cumulative food and water intake and the development of body weight after the two different treatments. Cumulative food intake was significantly reduced during night 1 and night 2 in the Zy group (Fig. 3b). In the third night as well as during day 1-3 no significant difference was observed. No significant changes were measured for cumulative water intake during all days and nights. In line with the depressed food intake, rats treated with Zy developed a reduction in body weight gain that became significant on day 3 and day 4 after the injection.

DISCUSSION

Zymosan a TLR2/6 agonist and activator of the innate immune system: Mammalian TLRs play a crucial role as sensors of infection and induce a rapid activation of the innate immune response. Several agonistic microbial molecules have been implicated in cell activation through TLR2 including Mycoplasma-specific diacylated lipopeptides and other bacterial lipoproteins, peptidoglycan as well as lipoteichoic acid from Gram-

positive bacteria, mycobacterial lipoarabinomanan, glycosylphoshatidylinositol anchored proteins of Trypanosoma cruzi, phenol-soluble modulin and finally the nonbacterial yeast agent Zymosan (Zy) (Dziarski and Gupta, 2005; Takeuchi *et al*., 1999; Takeda *et al*., 2003; Muhlradt and Frisch, 1994; Muhlradt *et al*., 1997; Underhill *et al*., 1999; Hajjar *et al*., 2001). One basis of the still existing specificity for such a broad range of PAMPs seems to be the agonist specific heterodimerization of TLR2 with either TLR1 or TLR6 (Ozinsky *et al*., 2000) and the different cytokine formation patterns resulting from distinct pathogenic microbial molecules (Plata-Salaman, *et al*., 1999).

Similar to the situation known for the Mycoplasma-specific diacylated lipopeptides MALP-2 and FSL-1, inflammatory responses to Zy seem to require heterodimerization of TLR2 and TLR6 (Ozinsky *et al*., 2000; Underhill, 1999; 2003). In the case of MALP-2 and FSL-1, activation of the TLR2/6-heteromer is finally leading to a pronounced formation of the proinflammatory cytokines TNF and IL-6 *in vitro* (Takeuchi *et al*., 2000, 2001) and *in vivo* (Hubschle *et al*., 2006). Identical mechanisms seem to apply to Zy-induced inflammation. After zymosan phagocytosis both TLR subtypes are recruited to macrophage phagosomes and the experimentally induced expression of inhibitory forms of either TLR2 or TLR6 does not inhibit the phagocytosis per se, but does block the activation of inflammatory signaling, e.g. NFkB activation and TNF secretion (Ozinsky *et al*., 2000; Underhill *et al*. 1999). Moreover, TLR2-deficient macrophages internalize Zy particles but fail to produce cytokines (Ganter *et al*., 2003). Besides macrophages, other immune-competent cells such as monocytes and mast cells seem to play an additional important role during Zy-induced inflammation, capable of producing and secreting proinflammatory cytokines in response to Zy (Von Asmuth *et al*., 1990; Underhill, 2003).

Proinflammatory cytokines, which can be induced by various PAMPs, are regarded as endogenous mediators of brain-controlled signs of illness (Dantzer, 2001, 2004). The aim of this study was to analyze changes in motor activity, food and fluid intake, body temperature and body weight as sickness behavior indices under the condition of nonseptic Zy-induced inflammation and to further to compare these changes with those induced by the two other TLR2/6 agonists MALP-2 and FSL-1.

Thermal responses induced by zymosan: Only little information is available on thermal responses induced by Zy under nonseptic, nonhypothermic conditions. Ip treatment with 10 mg kg^{-1} Zy in rats led to a significant elevation of T_{abd} for a period of about 4 h on the day of injection, which can be considered as a moderate febrile response. Thereafter T_{abd} did not differ any more from the temperature values observed in the control group over the entire time of analysis. At the subneutral ambient temperature of 23.5°C used for this study, no sign of hypothermia was observed after Zy injection. We therefore consider this treatment as a nonseptic Zy-induced systemic inflammatory response. In general, thermal responses to Zy seem to depend on the dose used. Shortly after the systemic treatment of a high dose (800-1000 mg kg^{-1}) severe hypothermia (ΔT of 6°C) was induced that lasted from hours up to one day (Volman *et al*., 2005) while systemic Zy treatment with low or medium doses (0, 5-150 mg kg^{-1}) induced no or a mild hypothermia (ΔT of 1°C) that lasted for around two hours (Cremeans *et al*., 2003; Li *et al*., 2002). Temperature data derived from TLR2/6 agonist-treated rats (MALP-2 and FSL-1) and made under identical experimental conditions as those recorded for Zy, revealed dose-dependent changes in T_{abd} with all doses inducing a febrile response (Hubschle *et al*., 2006). Only the lowest FSL-1 dose (10 μg kg^{-1}) did not induce any sign of hypothermia and showed a similar febrile response as those shown here for Zy with a temperature peak occurring around 300 min after the treatment. In line with our data, a careful investigation of the thermal response to Zy in guinea pigs was leading to the conclusion, that Zy has inherently pyrogenic properties, but only when given in low doses, so that no concomitant activation of the complement cascade could have occurred (Li *et al*., 2002). Indeed, Zy has many proposed ways of inflammatory action using several inflammatory mediators, amongst them Zy-induced cytokines and oxygen radicals as well as the activation of the complement system (Volman *et al*., 2005). As for the induction and maintenance of a febrile response it seems to be likely that proinflammatory cytokines are important Zy-induced fever mediators. This can be assumed from our in vivo temperature data in rat with other TLR2/6 agonists showing elevated levels of circulating proinflammatory cytokines, such as TNF and IL-6, during the time course of fever (Hubschle *et al*., 2006). Furthermore mice deficient in IL-1ß exhibited decreased Zy-induced circulating IL-6 levels when compared with their wild-type controls (Fantuzzi *et al*., 1997) and TNF blockade with anti-TNF antibodies was effective in reducing IL-6 levels (Von Asmuth *et al*., 1990). As for the regulation of fever, a correlation of the febrile response with elevated levels of circulating proinflammatory cytokines has been documented in detail for other PAMPs such as Lipo Poly

Saccharide (LPS) from Gram-negative bacteria and the minimally active subunit of peptidoglycan, e.g., Muramyl Dipe Ptide (MDP) (Kluger, 1991).

Other brain-controlled sickness responses induced by zymosan: The term sickness behavior describes a pattern of behavioral changes that can accompany the state of an activated immune system. These changes are due to the effects of proinflammatory cytokines on distinct brain target cells (Dantzer, 2001, 2004). It has further been suggested that a production of brain-intrinsic cytokines and a subsequent activation of brain-intrinsic cytokine receptors trigger long-lasting behavioral effects, which follow the treatment with various bacterial or nonbacterial PAMPs (Dantzer, 2001, 2004). To still guarantee specific reactions to a broad range of PAMPs, different brain cytokine formation patterns can be induced from distinct pathogenic microbial molecules (Plata-Salaman *et al*., 1999).

Sickness behavior induced by Zy has received little attention. To our knowledge only a single study performed in female rats investigated Zy-induced sickness behavior. This study did not use modern telemetric equipment for online recording of behavioral changes and was aiming at slightly different questions, namely at phenomena of tolerance and sensitization between Zy and LPS (Cremeans *et al*., 2003). The present study was performed to reevaluate sickness behavior during systemic Zy-induced inflammation in telemetrically monitored male rats under nonseptic conditions, not accompanied by any sign of hypothermia. In addition, emphasis was directed towards the comparison of behavioral aspects that were induced by Zy as compared to the two Mycoplasma-specific TLR2/6 agonists MALP-2 and FSL-1 (Hubschle *et al*., 2006).

Zy-induced inflammation has been reported to be associated with a reduction in food intake in mice and rats (Fantuzzi *et al*., 1997; Cremeans *et al*., 2003). In agreement with these studies systemic treatment with 10 mg kg^{-1} Zy induced a significant reduction of food intake during night 1 and night 2. In contrast drinking behavior was not significantly influenced. As a result of depressed food intake but unchanged drinking, rats treated with Zy developed a reduction in body weight gain that became significant on day 3 and day 4 after the injection. A tendency towards a generally lower motor activity was observed in Zy-treated animals, however, the values became significantly depressed only during night1 and night3. Treatments with the Mycoplasma-specific TLR2/6 agonists MALP-2 and FSL-1 also induced a significant depression of motor activity, anorexia and additional adipsia (Hubschle *et al*., 2006).

The specific role of endogenous inflammatory mediators involved in these behavioral depressions observed during TLR2/6 agonist-induced inflammation is not understood. However, first answering clues derive from our study suggesting the involvement of circulating IL-6 and TNF (Hubschle *et al*., 2006) and from studies using mice deficient in IL-1ß and TLR2. Anorexia which was induced by treatment with MDP, the minimally immunological active subunit of peptidoglycan from Gram-positive bacteria and an activator of TLR2, was attenuated in TLR2-deficient mice (Von Meyenburg *et al*., 2004), demonstrating that TLR2 is involved in the signaling pathway of MDP-induced anorexia. On the other hand, Zy-induced anorexia was not affected in IL1ß-deficient mice when compared with the wild-type controls (Fantuzzi *et al*., 1997) suggesting that this proinflammatory cytokine is not involved in the signaling pathway of Zy-induced anorexia. Nevertheless, at the beginning of the inflammatory process the activation of TLR2 and TLR6 seems to be a prerequisite for the manifestation of Zy-induced brain-controlled illness responses. Therefore we hypothesize that Zy-induced anorexia and depressed motor activity result from activation of cellular pathways involved in the TLR2/6-dependent innate immune response.

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