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Pyrogenicity of CpG-DNA in mice: role of interleukin-6, cyclooxygenases, and nuclear factor-κB

Wieslaw Kozak, Sylvia Wrotek, and Anna Kozak

1. Nicolaus Copernicus University, Institute of General and Molecular Biology, Department of Immunology, Torun, Poland; and 2. University of Georgia, Athens, Department of Clinical Pharmacy, Medical College of Georgia, Augusta, Georgia

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First published November 17, 2005; doi:10.1152/ajpregu.00408.2005.—Bacterial DNA containing unmethylated cytosine-phosphate-guanosine motif (CpG-DNA) has been identified as a pathogen-associated molecular pattern, which is recognized by Toll-like receptors and activates immune cells to produce cytokines. The aim of the study was to characterize the ability of CpG-DNA to induce fever in mice. Intravenous administration of unmethylated CpG-DNA 1826 triggered an elevation of body temperature (Tb) lasting several hours. The magnitude of Tb elevation increased with an increase of dose of the oligonucleotide (administered in a range from 0.01 mg/kg to 1.0 mg/kg). A fever-like increase of Tb in mice was partially dependent on IL-6, as IL-6 deficient mice responded with reduced fever to the CpG-DNA 1826. Meloxicam and sulindac sulfide, inhibitors of cyclooxygenases, reduced fever in mice challenged with CpG-DNA 1826, indicating that the process may also depend on prostaglandin. In fact, plasma levels of prostaglandin E2, as well as IL-6, increased at 4 h postinjection of CpG-DNA 1826 into mice. These data demonstrate that the pathophysiological mechanism of the increase of Tb induced by CpG-DNA 1826 is similar to fever induced by LPS. Both LPS and CpG-DNA 1826 failed to produce elevation of Tb in mice deficient for a nuclear factor-κB (NF-κB) gene, further supporting the hypothesis that the two pyrogens provoke fever, using the same components of the cellular signaling metabolism. However, parthenolide, an inhibitor of I-κB kinase reduced fever due to CpG-DNA 1826, and did not affect fever to LPS, suggesting that the two structurally dissimilar pyrogens may affect different intracellular pathways leading to the upregulation of NF-κB. In support of this hypothesis, we demonstrate that C3H/HeJ mice, known to exhibit a mutation in the Toll-like receptor-4 gene, do not respond with fever to LPS. They respond, however, with fever after injection of CpG-DNA 1826. We conclude that bacterial DNA shares with components of the bacterial wall the capacity to elicit fever and may, consequently, be part of a novel class of exogenous pyrogens.

FEVER IS A CARDINAL MANIFESTATION of infectious diseases. The current concept of fever physiology suggests that the febrile response is orchestrated by a number of the host cell-derived molecules. Among these factors are cytokines such as IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and IFN-γ, collectively termed endogenous pyrogens (25, 28). Cytokines, in turn, downstream of the mechanism of fever, trigger 1) liberation of the arachidonic acid from membrane phospholipids, 2) activation of cyclooxygenase (COX), and 3) subsequent production of prostanoids. It has been established that COX exists in two isoforms known as constitutive (housekeeping) COX-1 and inducible COX-2 (56). It is thought that induction of the expression of COX-2 by pyrogenic cytokines and the resulting excessive generation of PGE₂, play a critical role in affecting the thermoregulatory centers in the hypothalamus to start the rise of body temperature (6, 20). This train of pathophysiological molecular events is initiated upon contact of the host with non-self-immunostimulatory agents termed exogenous pyrogens (25).

In spite of the large number of pathogenic microbes and the ubiquity of the febrile response, only a few bacterial agents have thus far been identified as exogenous pyrogens. Research into the structure of the microbial pyrogens has established that fever-inducing agents are mostly located on the outer bacterial surface or in the cell wall (12). Immunostimulatory structures such as LPS, peptidoglycans, porin complexes, lipoteichoic acid, and lipourabinnomannans constitute a major class of the pyrogens of gram-negative, gram-positive, and mycobacterial origin (12, 17, 39). Another group of bacterial pyrogens includes staphylococcal and streptococcal proteinaceous superantigens, potent T cell mitogens and inducers of the pyrogenic cytokine release (44). Also a fever-inducing lipoprotein from mycoplasma has recently been characterized (19). However, the pyrogenic activity of LPS has thus far been the most extensively and thoroughly studied in the laboratory setting (see e.g., 25 and 26), and the LPS-induced fever has been regarded as a standard in research focusing on the molecular mechanisms of the febrile responses provoked by pyrogenic agents (6).

It has recently been reported that synthetic oligodeoxynucleotide (ODN) of bacterial DNA injected subcutaneously into the sheep and cattle can also induce transient fever (40), indicating that bacterial DNA may form an additional specific class of exogenous pyrogens. The mechanism of this fever-like response to bacterial DNA has not yet been investigated. However, it was demonstrated that bacterial DNA is a potent activator of the immune system (2, 38, 51). It is thought that presence of cytosine-phosphate-guanosine dinucleotide not methylated at the cytosine base, in a particular base context named the cytosine-phosphate-guanosine (CpG)-motif (30) may be accountable for the immunostimulatory effects of

Address for reprint requests and other correspondence: W. Kozak, Dept. of Immunology, Institute of General and Molecular Biology, Faculty of Biology and Earth Sciences, Nicolaus Copernicus Univ., 9 Gagarina St., 87–100 Torun, Poland (e-mail: wkozak@iob.uni.torun.pl).

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bacterial DNA. Consequently, several studies confirmed that ODNs containing unmethylated CpG islands (CpG-DNA) mimic the stimulatory effects of bacterial DNA (31, 57). It has been reported that CpG-DNA stimulate secretion of the proinflammatory cytokines including IFN-γ, TNF-α, and IL-6 from cultured leukocytes (24, 57), as well as in vivo (55). Induction of COX-2 and subsequent elevation in the generation of PGE₂ in mouse spleen cells incubated with CpG-DNA has also been demonstrated (11). Interestingly, studies using cultured cells revealed that murine and human cells differ in their preference for the core CpG-motif. Human cells prefer CpG-motifs containing more than one CG and the core sequence GTCGTT, whereas mouse cells respond better to CpG-DNA containing the core sequence GACGTT (4, 5, 18, 58).

CpG-DNA and LPS constitute the pathogen-associated molecular patterns (PAMPs) recognized by Toll-like receptors (TLRs). Different TLRs, however, are engaged in the recognition of these two bacterial immunostimulating agents. LPS is recognized by TLR4 (43, 45), whereas unmethylated CpG-DNA signals via TLR9 (53, 54). Nevertheless, it is thought that a common downstream pathway operates in the signal transduction via TLRs, involving the myeloid differentiation factor 88 (MyD88)-dependent and MAPK-dependent upregulation of the nuclear factor-κB (NF-κB) (22, 54). Intracellular upregulation of NF-κB, on the other hand, is necessary for the induction of the synthesis of cytokines, including pyrogenic cytokines (3), suggesting that NF-κB may be central to the generation of fever. In support of this, it has recently been demonstrated that nonspecific inhibitors of NF-κB can reduce LPS-induced fever in rabbits (33). There are reports, however, suggesting also the existence of a MyD88-independent pathway for the LPS-induced upregulation of NF-κB and expression of LPS-inducible genes (22, 23). Considering the resemblance in the immunologic activities of CpG-DNA and LPS and in the cell-signaling pathways triggered by these two bacterial factors, we investigated the role of NF-κB, IL-6, and cyclooxygenases in fever provoked by the injection of CpG-DNA.

MATERIALS AND METHODS

Animals

The following male mice from the Jackson Laboratories (Bar Harbor, ME) were used throughout the experiments: 1) specific pathogen-free C57BL/6j mice; 2) B6:129S2-Irge1KmJ/T (IL-6−/−); stock number 002254), mice, and control wild-type (IL-6+/+) F2 generation of 129Sv(ev)/C57BL6 hybrid mice; 3) B6:129P-Nfkb1tm1Boi (NF-κB−/−; stock number 002849) mice, and control wild type (NF-κB+/+) B6:129F2J, stock number 100903 mice; 4) endotoxin-resistant C3H Heston (C3H/HeJ; symbol Tlr4−/−; stock number 000659) mice.

Animal Care

All mice were 3–5 wk of age upon arrival. The care and treatment of the mice were conducted, as approved by The Medical College of Georgia (MCG) Animal Care and Use Committee. Mice were kept in the MCG specific pathogen-free facility, where the animal experiments were performed. The mice were housed in individual plastic cages and maintained in a temperature/humidity/light-controlled chamber set at 29 ± 1°C, 12:12-h light-dark cycle, with light on at 0600. Rodent laboratory chow (Teklad Rodent Diet, W 8604) and drinking water were provided ad libitum. Ten days after shipment, the mice were implanted under sterile conditions with biotelemetry devices to monitor body temperature and motor activity (see below). The mice were usually in their 9th wk of age during the beginning of the experimentation.

Injections and handling for the experimentation were made at 9:00 AM to avoid an influence of the circadian rhythm on the responses. All injections were made in the animal chamber to avoid exposing the mice to transportation stress and change of ambient temperature (see Refs. 27 and 49). Immediately after the procedure (i.e., handling and injection, depicted in the figures by arrows), each animal was returned to its home cage for temperature recording.

Body Temperature Measurement

Deep body temperature (Tb) of the mice was measured with an accuracy of ±0.1°C using battery-operated miniature biotelemeters (model VMFH MiniMitter, Sunriver, OR) implanted intra-abdominally (for details see Refs. 27–29). Recordings were made at 5-min intervals in unstressed, freely moving mice kept in their individual home cages, using a peripheral processor (Datquest III System) connected to an IBM personal computer. The system allowed also for monitoring a motor activity of the mice to assess their progress in recovery from the surgery. The mice were monitored daily following surgery, and usually, they exhibited normal, regular circadian rhythm in Tb and motor activity on day 4 postsurgery. Experimentation started 10 days after the surgery and were preceded by a 3-day monitoring of the regular rhythm of Tb in undisturbed mice.

Reagents and Injections

3′-Biotin-modified ODNs: CpG-DNA 1826 (TTCAGCTCTTTTCTGAGCTTTGACGCT) and non-CpG-DNA 1826-GC (TTCAGCTCTTCTTCTGAGCTTTGACGCT) were synthesized by Qiagen (Hilden, Germany). Calf thymus DNA was purchased from Sigma (St. Louis, MO). All ODNs and DNA were further purified by extraction with phenol:chloroform: isomyl alcohol (25:24:1) as recommended (11). Contents of LPS in DNA preparations was <1.5 ng/ml as assayed with Limulus Amoebocyte Lysate Assay (BioWhittaker, Walkersville, MD). Resultant extracts were dissolved in apyrogenic saline for injections. Doses of respective oligonucleotides and calf thymus DNA were injected intravenously into the tail vein of an animal that was placed into a custom-made restrainer during injection.

LPS derived from Escherichia coli (0111:B4, Sigma Chemical, St. Louis, MO) was dissolved in sterile 0.9% sodium chloride (saline) at a stock concentration of 2 mg/ml and kept frozen (−20°C). Before use and dilution to desired concentration, a portion of the stock was preheated to 37°C, vortexed, and briefly sonicated. LPS was injected intravenously into the tail vein at a dose of 0.05 mg/kg in an injection volume of 0.05 ml/mouse. Pyrogen-free saline was used for control injections. Mice were restrained and not anesthetized during the LPS and/or saline intravenous injections.

Salindac sulfide and meloxicam (Biomol, Plymouth Meeting, PA), inhibitors of cyclooxygenases (13) were dissolved in DMSO at a concentration of 5 mg/ml each. The agents were administered intraperitoneally in separate experiments at a dose of 10 mg/kg each (injection volume −0.06 ml/mouse; the same volume of DMSO alone was used as control injections). Agents were injected intraperitoneally 30 min before administration of pyrogens.

Parthenolide (Sigma-Aldrich; catalog number P0667), found in Feverfew (Tanacetum parthenium L.), a sesquiterpene lactone that inhibits the IkB kinase complex (32), was prepared as an aqueous solution in 0.01 M anhydrous sodium carbonate. A dose of 2 mg/kg of parthenolide (administered intraperitoneally 30 min before injections of the pyrogenic factors) was selected for the present experiments, because this dose prevented carrageenan-induced paw edema in rats and mice (21).
Plasma Assays

Specific pathogen-free C57BL/6J mice were used in experiments to measure circulating IL-6 and PGE2. At the time of injections (0 h) and 4 h postinjection (LPS, calf thymus DNA, CpG-DNA 1826, non-CpG-DNA 1826-GC, and/or appropriate vehicle), separate groups of mice were anesthetized (inhaled isoflurane), and heparinized blood was collected via cardiac puncture. Plasma samples were stored at −80°C until the assays. IL-6 levels were determined by a standard sandwich ELISA kit from R&D Systems (Minneapolis, MN; cat. no. M6000; sensitivity of assay 3 pg/ml, range 15–1,000 pg/ml). Colorimetric changes of enzyme substrates were detected at 450 nm wavelength, using an ELISA plate reader EL-312 (Bio-Tek Instruments). Plasma PGE2 levels were determined in duplicate using a highly sensitive colorimetric assay kit from R&D Systems (cat. no. SE0100; sensitivity assay 36.2 pg/ml, range 39–5,000 pg/ml), according to the manufacturer’s instruction. The wells were read at 670 nm with plate reader as above. Indomethacin (in a ratio of 0.1 ml of 0.01 M indomethacin per 1 ml of blood), in addition to heparin, was added to the blood samples used for PGE2 measure.

Data Analysis

Values are reported as means ± SE. Five-minute temperature recordings were pooled into 0.5-h averages for presentation. Data were analyzed using Statview SE+Graphics (Abacus Concepts, Berkeley, CA). ANOVA with repeated measures was used to determine differences among groups in patterns of temperature changes over time. ANOVA followed by Scheffé’s pairwise comparisons was used to test for statistical differences among groups at individual time points. Data of plasma levels of IL-6 and PGE2 were analyzed by Student’s t-test in addition to ANOVA. Differences were considered significant when P < 0.05. Data were plotted using KaleidaGraph for Macintosh (Synergy Software, Essex Junction, VT).

RESULTS

CpG-DNA 1826 Induces an Increase of Tb in Mice

Effect of CpG-DNA 1826 on changes of Tb in C57BL/6J male mice is illustrated in Fig. 1A. Three doses of the oligonucleotide and control saline were injected intravenously into the separate groups of mice. Both the elevation of Tb and the duration of fever were dose-dependent, with the lowest and shortest response after an injection of CpG-DNA 1826 at a dose of 0.01 mg/kg. Because the fever induced by the dose of 1.0 mg/kg was not different from that promoted by the dose of 0.1 mg/kg, the lower dose was selected for further experiments. Elevation of Tb after injection of the oligonucleotide at a dose of 0.1 mg/kg peaked at 5 h postinjection, and the fever lasted ~8 h postinjection.

Injections of the calf thymus DNA, as well as non-CpG-DNA 1826-GC (0.1 mg/kg each) into separate groups of C57BL/6J mice did not provoke any significant alterations in Tb (Fig. 1B).

Regardless of the treatment, all mice responded with a sharp increase in Tb to handling and injections (at the time marked by an arrow). This response is particularly well seen in Fig. 1B, which depicts responses of mice injected with nonpyrogenic DNA and saline. In mice injected with a fever-inducing CpG-DNA 1826 (Fig. 1A) this response is masked by febrile elevation of Tb, which starts presumably within 1 h postinjection after an intravenous injection of the pyrogen. As can be seen in Fig. 1A, mice injected with saline, a vehicle for CpG-DNA 1826, displayed a gradual decrease in Tb to normal daytime level after the response to handling and injection.

Effect of CpG-DNA 1826 on Plasma IL-6 Levels in Mice

Mice injected with saline (control vehicle) and with non-CpG-DNA 1826-GC (0.1 mg/kg) did not show any significant elevation of IL-6 at 4 h postinjection compared with the levels measured at time 0 h (Fig. 2). Also, injection of calf thymus DNA did not induce changes in the levels of IL-6 (data not shown). In contrast, however, injection of CpG-DNA 1826 (0.1 mg/kg) provoked a 10-fold elevation of the plasma IL-6 (from 24 ± 14 pg/ml at time 0 h to 234 ± 57 pg/ml at 4 h postinjection). Injection of LPS (0.05 mg/kg for a positive control) induced an even greater increase of circulating IL-6 in the mice, from 12 ± 7 pg/ml at time 0 h to 467 ± 64 pg/ml at time 4 h.
To test the role of IL-6 in the generation of fever after injection of CpG-DNA 1826, we used genetically engineered mice lacking the ability to produce the cytokine upon inflammation. As can be seen from Fig. 3A, during the first 4 h postinjection, Tb was essentially the same in wild-type and IL-6−/− mice treated with the oligonucleotide, and then it declined in the IL-6-deficient mice. A significant reduction of fever in IL-6−/− mice occurred after 4 h from the injection of CpG-DNA 1826. One can conclude from Fig. 3A that duration of fever in IL-6−/− mice was ~50% shorter compared with the time course of fever in wild-type mice. Lack of IL-6, on the other hand, resulted in a complete prevention of fever due to LPS (Fig. 3B).

CpG-DNA 1826 Provokes an Elevation of Plasma PGE2

Administration of CpG-DNA 1826, as well as of LPS, induced significant elevation of plasma PGE2 at 4 h postinjection compared with injections of saline and non-CpG-DNA 1826-GC (Fig. 4).

To test the role of COX in the fever induced by CpG-DNA 1826, we applied meloxicam and sulindac sulfide (Fig. 5, A and B, respectively). Mice treated with meloxicam and then 30 min later injected with oligonucleotide revealed an undisturbed fever for ~2.5 h from the injections. After that time the Tb of meloxicam-treated mice decreased reaching a normal level, that is, the level of Tb of mice injected with saline and meloxicam, while those treated with vehicle (DMSO) and CpG-DNA 1826 maintained febrile level for ~9 h postinjection (Fig. 5A). Sulindac sulfide also reduced fever in mice (Fig. 5B). C57BL/6J mice pretreated with sulindac sulfide did not generate fever to the oligonucleotide until ~3.5 h postinjection (Fig. 5B). Starting 4 h postinjection of CpG-DNA 1826, of sulindac sulfide-treated mice increased to the level recorded for vehicle-treated mice.

Meloxicam and sulindac sulfide at a dose of 10 mg/kg did not affect normal Tb of the mice. Higher doses (5× and 10× higher), on the other hand, provoked a significant dose-dependent decreases of normal Tb in mice and, therefore, were not tested in the context of fever induced by CpG-DNA 1826 (data not shown). Both inhibitors of COXs were injected as DMSO solutions, at volumes that did not exceed 0.06 ml/mouse. This volume of DMSO did not affect normal Tb of mice compared with vehicle-treated mice.
with the changes in temperature of the animals injected intraperitoneally with sterile saline (data not shown).

Pretreatment with meloxicam completely suppressed fever to LPS in mice. Sulindac sulfide, on the other hand, unlike the suppression of the oligonucleotide-induced fever presented above, did not exhibit any significant influence on the LPS-provoked fever in mice (data not shown). These data suggest that LPS and CpG-DNA 1826 may induce fever via different signal transduction mechanisms, which finally converge on the involvement of IL-6 and COXs, however, in different proportions and relations. As we pointed out in the introduction, LPS and CpG-DNA 1826 are ligands for different TLRs. We hypothesized, therefore, that the mechanism of fever could be induced by different TLRs recognizing different PAMPs. After the recognition, however, the pyrogens may trigger either a common intracellular signaling pathway or several different signal transduction pathways, resulting in fever. To test this hypothesis, in the following studies we made use of C3H/HeJ mice mutant for TLR4, parthenolide, and mice deficient for NF-κB.

Fever in C3H/HeJ Mice

There was a remarkable difference in the responses of C3H/HeJ mice to the two pyrogenic compounds (Fig. 6). Animals injected with LPS did not develop any fever, and their postinjection time course of Tb was similar to that of mice injected with saline. Injection of CpG-DNA 1826 into C3H/HeJ mice, on the other hand, provoked an increase of Tb lasting 7 h postinjection (Fig. 6). Pretreatment with meloxicam (10 mg/kg) reduced the elevation of Tb in C3H/HeJ mice given CpG-DNA 1826 (data not shown). As can be seen in Fig. 6, regardless of the injection the mice always responded with an elevation of Tb to handling.

Effect of Parthenolide on Fever in Mice

Parthenolide injected intraperitonely at a dose of 2.0 mg/kg 30 min before CpG-DNA 1826 significantly reduced fever in C57BL/6J mice (Fig. 7A). In contrast, however,
treatment with parthenolide at this particular dose, as well as
with a dose 5-fold higher (10 mg/kg), did not affect the
elevation of Tb in mice following injection of LPS (data not
shown). Furthermore, pretreatment with parthenolide inhibited
an increase of plasma IL-6 in mice given CpG-DNA 1826,
while it did not influence the cytokine increase after injection
of LPS (Fig. 7B). These results may suggest that signaling via
NF-κB is specific for the pyrogenic action of CpG-DNA and
not for LPS. To test this hypothesis, we used NF-κB-deficient
mice.

NF-κB KO Mice Are Resistant to the Pyrogenic Effects of
CpG-DNA 1826 and LPS

Results of the study using mice deficient for NF-κB and their
wild-type control are summarized in Fig. 8. Postinjection
changes in Tb are plotted against a normal daytime temperature
recorded for undisturbed NF-κB KO mice during a day before
the experimentation (shown as control day; solid line with open
squares).

Circadian variations of Tb in mice were unaffected by a
deficiency in NF-κB; diurnal rhythms of Tb in NF-κB −/−
and wild-type mice were virtually the same (12-h average Tb
computed for the daytime was 36.67 ± 0.09°C for NF-κB −/−
and 36.63 ± 0.11°C for wild-type mice, and during nighttime
was 37.53 ± 0.12°C and 37.58 ± 0.14°C, respectively; data
not shown). Lack of NF-κB had also no influence on a rapid
and brief increase of Tb in response to handling and injection
(Fig. 8). As can be seen in Fig. 8, NF-κB KO mice injected
with CpG-DNA 1826 did not generate fever, and postinjection
changes of their Tb did not differ from that recorded a day
before the experimentation. Wild-type mice injected with oli-
gonucleotide, on the other hand, developed fever with a pick of
Tb at ~5 h postinjection.

Injection of LPS into the wild-type mice provoked fever that
peaked at night hours postinjection (data not shown). In con-
trast, the lack of NF-κB resulted not only in the prevention of
fever to LPS, but, above all, it precipitated a significant and
long-lasting drop of Tb after injection of LPS (Fig. 8; solid
triangles). This decreasing in Tb of NF-κB KO mice treated
with LPS lasted for more than 20 h postinjection, and Tb of
these mice returned to normal next morning (data not shown).

DISCUSSION

Numerous preclinical studies revealed an adjuvant potency
of CpG-DNA for the induction of systemic immune responses
against a variety of antigens, including responses against tumor cells (1, 30, 31, 35). In addition, Nichani et al. (40) have recently reported that intramuscular injection of a dose of CpG oligodeoxynucleotide induced a fever-like transient increase of body temperature in sheep and cattle. The most frequently studied bacterial pyrogens such as LPS can also activate multiple arms of the immune system after injection into the laboratory animals (see e.g., 25, 26). These observations suggested to us that CpG-DNA and LPS may share the capacity to initiate fever during natural infections.

Data presented in our report demonstrate similarities in certain aspects of fevers in mice challenged with CpG-DNA 1826 and LPS. Similar to LPS, injection of CpG-DNA 1826 also provoked elevation of circulating IL-6 and PGE2 in mice. As was pointed out in the introduction, involvement of PGE2 and IL-6 as endogenous mediators of the febrile response to LPS has been well documented. Both mediators are also involved in fever generated by CpG-DNA, albeit in slightly dissimilar proportions compared with the fever induced by LPS.

Meloxicam completely suppressed fever in mice challenged with LPS, whereas in mice injected with CpG-DNA 1826, the reduction of fever occurred not sooner than 2.5 h postinjection of the oligonucleotide. Meloxicam is an enolcarboxylamide-related to piroxicam (41). In various assays, meloxicam has been estimated to be between 3- and 77-fold selective for COX-2 over COX-1 (41). For example, in studies using LPS-stimulated guinea pig peritoneal macrophages meloxicam displayed approximately fivefold higher inhibitory potency for COX-2 than for COX-1 (16). Furthermore, in a concentration-wise, meloxicam was 3 times more potent than indomethacin, and 3,500 times than furbiprofen in inhibiting COX-2 (16). This limited selectivity has led meloxicam to be described as a preferential COX-2 inhibitor (16). These results indicate that LPS-induced fever is more dependent on products of COX-2 than is the fever induced by CpG-DNA 1826.

Another COX inhibitor used in our studies, sulindac sulfide, failed to affect fever in mice injected with LPS. It reduced fever, however, in mice challenged with CpG-DNA 1826. In contrast to the action of meloxicam, the reduction of fever in mice pretreated with sulindac sulfide occurred immediately postinjection of the oligonucleotide, and it lasted for a relatively short period of 3 h after the injection. Sulindac sulfide is considered a nonselective inhibitor of COXs (13). Nevertheless, on the basis of the results of studies evaluating the biochemical efficacy of sulindac sulfide in inhibition of the two COX isomers, the agent can be regarded as a preferential COX-1 inhibitor. For example, in the human whole blood assay, the drug was 10 times more potent in inhibiting COX-1 (IC50 = 1.02 micromol) than COX-2 (IC50 = 10.43 micromol) (7). In the murine microsomal enzyme preparation assay, sulindac sulfide inhibited COX-1 activity with IC50 = 0.3–0.5 micromol, and the activity of COX-2 with IC50 = 11–14 micromol and was ~10 times more potent in inhibiting COX-1 than indomethacin and piroxicam (37). Besides biochemical efficacy, one has to consider also a pharmacokinetics of the drugs used in the in vivo experiments. Both sulindac sulfide (13) and meloxicam (8) injected into mice displayed ~8 h half-time in the circulation and tissues, indicating that 30-min pretreatment used in our studies was sufficient to maintain saturation of the tissues with inhibitors.

Thus, analyzing the data presented in our report in the context of the biochemical preference of the two inhibitors, we conclude that LPS-induced fever in mice is entirely dependent on the induction and activation of COX-2. This conclusion is supported by findings by Li et al. (34), who have shown that mice lacking an active COX-2 gene failed to produce fever after injection of LPS, while those deficient for COX-1 showed fevers after administration of LPS. Considering involvement of the two COX isomers in fever in mice challenged with CpG-DNA, we hypothesize that this fever is multimediating and may consist of two time-course parts (phases), each depending on a different COX isomer. Namely, an early part (or early phase) of fever induced by CpG-DNA 1826 seems to be dependent on the activity of COX-1, whereas COX-2 may be accountable for a late part (or late phase) of fever. This feature may distinguish fever induced by CpG-DNA 1826 from fever provoked by LPS in mice and may have an impact on further differences in responses of mice to the two structurally dissimilar pyrogens.

Conclusion regarding a multimediating fever caused by CpG-DNA in mice is further substantiated by results of the experiments using parthenolide and IL-6 knockout mice. That our report showed a lack of fever in IL-6−/− mice challenged with LPS extends previous findings, which found mice deficient in IL-6 gene failed to generate fever to LPS (10, 28, see also 48). In contrast, IL-6−/− mice injected with CpG-DNA 1826 responded with a partial fever, which corresponded to a time course of the early part. Therefore, we conclude that this component of CpG-DNA-induced fever in mice does not.

Fig. 8. Changes of Tb of wild type (control) and NF-κB deficient (NF-κB KO) mice injected with CpG-DNA 1826 and LPS as shown. Values are expressed as means ± SE (n = sample size). Data are plotted against changes in average Tb recorded over a day before the experimentations of all NF-κB KO mice (control day; solid line with open squares). Significant difference is depicted as follows: the letter “a” indicates difference (P < 0.01) between wild-type and NF-κB KO mice both injected with LPS; “b” shows difference (P < 0.05) between groups of NF-κB KO mice treated with LPS and CpG-DNA 1826; and “c” depicts a difference (P < 0.05) between wild-type and NF-κB KO mice both injected with CpG-DNA 1826.
depend, at least solely, on the generation of IL-6. Parthenolide prevented elevation of plasma IL-6 in mice treated with CpG-DNA 1826. Consistently, it reduced mostly the late part of fever, supporting a conclusion that, indeed, an early part of CpG-DNA-induced fever in mice may relate to endogenous factor(s) other than IL-6. In this context, however, a direct action of CpG-DNA on the thermoregulatory centers during the early part of fever cannot be ruled out, as TLR9 mRNA expression in the mouse hypothalamus has been detected (42).

Laboratory animals with larger body mass, such as rats, guinea pigs, and rabbits, usually respond with biphasic fever to the injection of LPS (46). Some laboratory data suggest that exhibition of the two phases of fevers in these species may also depend on different cyclooxygenases; however, the data have not yet been conclusive. For example, Zhang et al. (59) have recently reported that intraperitoneal injection of SC-236, a COX-2 inhibitor blocked LPS-induced fever in rats, whereas injection of SC-560, an inhibitor of COX-1, resulted in profound hypothermic response to LPS. However, Dogan et al. (15) demonstrated that an inhibitor of COX-1 (valeryl salicylate) completely blocked an early hypothermia-like response of rats to LPS while SC-236, an inhibitor of COX-2, had no effect on the initial stages of fever, and it inhibited the late phase of fever in rats. Also in studies by Cao et al. (9), treatment of rats with a selective COX-2 inhibitor (NS398) prevented fever after injection of LPS. In studies by Steiner et al. (52) and Roth et al. (47), pretreatment of guinea pigs with COX-1 inhibitor (SC-560) did not affect febrile response to LPS, while injection of COX-2 inhibitors (nimesulide and meloxicam) attenuated the fever. Although these data suggest relative roles of COX-1 and COX-2 in the thermoregulatory responses to LPS in rats and guinea pigs, the exact nature of this relation is not clear. Recently, Ivanov and Romanovsky in their review article (20) have thoroughly discussed data indicating involvement of COX-1 and COX-2 in different phases of the time course of fever. Our data contribute to these studies and support a hypothesis that the relative role of COX-1 and COX-2 in various parts (phases) of fever may be species-specific and may depend, at least in mice, on the type of pyrogen. The resulting conclusion may also be affected by and related to the pharmacological and biochemical efficacy of the inhibitor used in the animals.

Our results using C3H/HeJ mice are in perfect agreement with data showing that LPS and CpG-DNA are recognized by different TLRs (see the introduction). C3H/HeJ mice mutant for TLR4 were unable to develop fever to LPS, while they maintained sensitivity to the pyrogenic effects of CpG-DNA 1826. Elevation of $T_b$ in C3H/HeJ mice given oligonucleotide was reduced by meloxicam, indicating that the process was prostaglandin dependent. On the basis of these data, we assume that C3H/HeJ mice expressed intact TLR9, which recognized CpG-DNA. After being recognized by their respective receptors, the two studied pyrogens may further signal into the cell to upregulate NF-$k$B. NF-$k$B appeared to be indispensable for the induction of fever to LPS and CpG-DNA 1826, as both pyrogens failed to trigger fever in NF-$k$B-deficient mice. However, these two pyrogens may utilize different downstream pathways for the upregulation of NF-$k$B, a hypothesis that is supported by the difference in the thermoregulatory responses to LPS between C3H/HeJ and NF-$k$B$^{-/-}$ mice, and by the results using parthenolide in mice. Namely, challenging mice deficient in NF-$k$B (and presumably possessing an intact TLR4 to recognize LPS) provoked a significant and long-lasting drop of $T_b$, whereas injection of LPS into C3H/HeJ mice lacking a functional Toll receptor recognizing LPS did not result in such a hypothermia-like effect. Injection of CpG-DNA 1826 into NF-$k$B$^{-/-}$ mice also did not result in the hypothermia-like decrease of $T_b$. We speculate, therefore, that these remarkable differences in the thermoregulatory responses to the two pyrogens in mice may reflect the differences in the ability of the pyrogens to affect various independent pathways of intracellular signaling. Supports for this supposition are 1) findings demonstrating that LPS may induce an upregulation of NF-$k$B and provoke an expression of LPS-inducible genes via a pathway that does not involve MyD88 protein, a classic pathway for the upregulation of NF-$k$B (22, 23), 2) accumulating evidence that NF-$k$B can also be the subject to the IkB-independent level of regulation (50), and 3) our data demonstrating that parthenolide, which is known to block the induction of NF-$k$B via MyD88 and IkB, did not affect fever in mice injected with LPS. We conclude, therefore, that these additional postrecognition pathways stimulated by LPS may be responsible for the drop of $T_b$ in mice deficient in NF-$k$B. The mechanisms underlying a decrease of $T_b$ in response to bacterial infection is of critical importance, because in humans, it contributes to a poor prognosis for survival (36).

Perspectives

Finding that bacterial DNA may form a pool of exogenous pyrogens adds a further chapter to the story focusing on the complex origin of clinical fevers. It is becoming clear that a number of structurally different microbial agents may provoke fever in the infected host. These observations suggest that nature has employed a significant repertoire of recognition mechanisms for the countless microbial products, as well as host-derived products, such as cytokines, as part of the fever-producing processes. Here, the question arises as to whether there is a unifying mechanism converging on the intracellular signal that leads to fever. Recently, such a unifying mechanism involving Toll-like receptors has been proposed (14). Our studies showing that TLR4/9 ligands trigger fever in mice, as well as recent data by Hubsche et al. (19), demonstrating that agonists of TLR2/6 induce fever in rats, seem to support this concept. Data presented in our report suggest, however, that activation of NF-$k$B may represent the major intracellular pathway for the fever induction and that possibly there are several independent intracellular pathways for the upregulation of NF-$k$B stimulated by various pyrogens. In this context, to provide additional data testing the concept of a unifying mechanism of fever, it is worthwhile to investigate fever provoked by administration of structurally different exogenous pyrogens, as well as endogenous mediators of fever, into mice deficient for genes encoding different TLRs.

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REFERENCES


