special communication

Methodology of fever research: why are polyphasic fevers often thought to be biphasic?

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Romanovsky, Andrej A., Vladimir A. Kulchitsky, Christopher T. Simons, and Naotoshi Sugimoto. Methodology of fever research: why are polyphasic fevers often thought to be biphasic? *Am. J. Physiol.* 275 (*Regulatory Integrative Comp. Physiol.* 44): R332–R338, 1998.—This study explains why the recently described triphasic lipopolysaccharide (LPS) fevers have been repeatedly mistaken for biphasic fevers. Experiments were performed in loosely restrained male Wistar rats with a catheter implanted into the right jugular vein. Each animal was injected with *Escherichia coli* LPS, and its colonic (T_c) and tail skin temperatures were monitored. The results are presented as time graphs and phase-plane plots; in the latter case the rate of change of T_c is plotted against T_c. At an ambient temperature (T_a) of 30.0°C, the response to the 10 μg/kg dose of LPS was triphasic, as is obvious from time graphs of T_c (3 peaks), time graphs of effector activity (3 waves of tail skin vasoconstriction), and phase-plane plots (3 complete loops). When the T_a was below neutral (22.0°C) or the LPS dose was higher (100 or 1,000 μg/kg), the time graph of T_c did not allow for the reliable detection of all three febrile phases, but the phase-plane plot and time graph of effector activity clearly revealed the triphasic pattern. In a separate experiment, LPS (10 μg/kg) or saline was injected via one of two different procedures: in the first group the injection was performed through the jugular catheter, from outside the experimental chamber; in the second group the same nonstressing injection was combined with opening the chamber and pricking the animal in its lower abdomen with a needle. In the first group the febrile response was obviously triphasic, and none of the phases was due to the procedure of injection per se (injection of saline did not affect T_c). In the second group the fever similarly consisted of three T_c rises, but it might have been readily mistaken for biphasic because the first rise was indistinguishable from stress hyperthermia occurring in the saline-injected (and needle-pricked) controls. We conclude that several methodological factors (dose of LPS, procedure of its injection, and T_a) have contributed, although each in a different way, to the common misbelief that there are only two febrile phases.

thermoregulation; lipopolysaccharides; skin vasoconstriction; stress hyperthermia; ambient temperature; restraint; body temperature oscillations; nonlinear dynamics; rats

IN A RECENT STUDY INVOLVING several different rat strains and several different lipopolysaccharide (LPS) preparations, we showed that the febrile response of the rat to a typical biphasic fever-inducing dose of LPS (10 μg/kg) consists of not two, but at least three, separate phases (16). The triphasic (or polyphasic, a more general term) pattern of experimental fevers was mentioned in the old literature (6) and occasionally noticed by colleagues in the field (J. M. Krueger, personal communication). Yet the recent physiological literature, although replete with examples of biphasic fevers, does not mention polyphasic febrile responses. What is the reason for this omission? The general goal of the present study is to analyze this question for the case of LPS fever in the rat. We propose that several methodological factors can modify the fever course and mask one or more febrile phases (rises in body temperature, T_b), thus changing the "correct" phase count.

The ambient temperature (T_a) is likely to be an important factor. It is well known that, at a T_a within or slightly above the thermoneutral zone, rats respond to LPS with a fever and that a hypothermic component appears in the response to LPS at T_a below thermoneutrality (17, 22). Our working hypothesis was that this hypothermic component can overlap one of the febrile phases, mask it, and thus interfere with the phase count. *Experiment 1* addresses this hypothesis.

Another obvious factor to consider is the dose of LPS. It is clear that the febrile response is monophasic if the LPS dose is small (just above apyrogenic); it is also clear that if the dose is slightly higher than one inducing a monophasic fever, the response is biphasic (11, 14, 22). It is not clear, however, why the polyphasic febrile responses to higher doses of LPS, i.e., ≥10 times greater than the monophasic fever-inducing dose (for a description of such responses, see Ref. 16), have been repeatedly assumed to consist of only two febrile phases (14, 15, 17). Could it be that, even within the range of doses causing a polyphasic response, there is a subrange in which a certain febrile phase becomes much less prominent than the others? If this is true, the less expressed phase can be easily overlooked. In *experiment 2* we investigate the effect of the dose of LPS on the pattern of the febrile response.

The effect of the method of LPS injection on the fever response may also be important. If the pyrogen is injected through a preimplanted catheter exteriorized from the experimental chamber (i.e., without disturbing the animal), the injection procedure per se does not induce any stress hyperthermia, as is obvious from the control experiments with saline injection (14–17, 22). If, however, the injection involves handling and prick-

ing the animal with a needle, stress-associated hyperthermia readily occurs (9, 12, 18). This stress-associated (injection induced) hyperthermia may overlap the authentic febrile response to the injected pyrogen, mask some part(s) of this response, and thus artifactually change the total number of febrile phases detected. In addition, a needle prick by itself has been recently shown to prolong the febrile response to the intraperitoneal administration of a low dose of LPS and perhaps to transform a monophasic fever into a biphasic one (21). *Experiment 3* is designed to test the hypothesis that the pyrogen administration technique may affect the investigator's judgment of the number of febrile phases observed.

METHODS

Animals and Surgical Preparation

Forty-six adult male rats of the Wistar strain (B & K Universal, Kent, WA) were used. The animals were initially housed three per box; after surgery, they were caged singly. The room was on a 12:12-h light-dark cycle; T_a was maintained at 22°C. Food [Teklad Rodent Diet (W) 8604, Harlan Teklad, Madison, WI] and water were available ad libitum. The animals were handled and weighed regularly. They were also habituated (5 training sessions, 3–4 h each) to a cylindrical restrainer that restricted their back-and-forth movements and prevented them from turning around; the same restrainer was used later in the experiment. Three days before the experiment, a catheter was implanted into the right jugular vein of each animal, as described in detail previously (16). The free end of the catheter was rolled into a coil and placed into a hollow polypropylene pedestal affixed to the skull; the pedestal was protected with a screw-on cap. On the day after the surgery the catheter was flushed with heparinized pyrogen-free saline (PFS). To obviate the possible effects of circadian rhythms, all experiments were started at the same time of day (between 0800 and 0900). To avoid the development of LPS tolerance, each animal was injected with LPS only once. At the end of the study, the rats were killed with an injection of pentobarbital sodium (20 mg/kg iv). The protocols were approved by the Institutional Animal Care and Use Committee.

Instrumentation

For an experiment, all animals were instrumented with homemade copper-constantan thermocouples for colonic (T_c; 9 cm from the anus) and tail skin (T_{sk}) temperature measurement. The thermocouples were connected to a data logger (model AI-24, Dianachart, Rockaway, NJ) and then to a personal computer. The animal was placed into its restrainer and transferred to a climatic chamber (Forma Scientific, Marietta, OH) set to a relative humidity of 50% and a T_a of 30.0°C (upper limit of the thermoneutral zone for rats) or 22.0°C (slightly cool environment). The exteriorized portion of the intravenous catheter was pulled through a wall port and connected to a syringe. After a 1-h stabilization period, the measurements were begun, and T_c, T_{sk}, and T_a were sampled every 2 min for 8 h.

Experimental Protocols

Experiment 1. In *experiment 1* we investigated how the T_a affects the febrile response to a 10 μg/kg dose of LPS. The animals were instrumented as described above, placed in the environmental chamber (set to a T_a of 22.0 or 30.0°C), and, 1 h after the recording was started, injected intravenously with the 10 μg/kg dose of LPS in PFS (1 ml/kg). The LPS used in all the experiments was from *Escherichia coli* 0111:B4, prepared by phenol extraction (lot no. 35H4086, Sigma Chemical, St. Louis, MO).

Experiment 2. Experiment 2 was designed to determine the effect of LPS dose on the shape of the T_c response. The T_a in the chamber was set at 30.0°C. The animals were injected with a 10, 100, or 1,000 μg/kg dose of LPS in PFS (1 ml/kg).

Experiment 3. In *experiment 3* we investigated how stress-associated hyperthermia interferes with the normal febrile course. The control rats were injected intravenously (via the preimplanted jugular catheter exteriorized through a wall port) with LPS (10 μg/kg) or PFS (1 ml/kg). The experimental rats received the same injection, but immediately before the injection the chamber was quickly opened and the animals were pricked in the lower abdomen with a 23-gauge needle.

Data Processing and Analysis

To evaluate the thermal response, the absolute value of T_c and its deviation from the mean T_c at the time of the injection (ΔT_c) were used. To evaluate the thermoeffector response of tail skin vasculature, the heat loss index (HLI) was calculated: $HLI = (T_{sk} - T_a)/(T_c - T_a)$; the HLI changes between 0 (maximal heat conservation due to skin vasoconstriction) and 1 (maximal heat loss due to skin vasodilation). To compare the responses between the groups, we performed a two-way ANOVA (repeated measures) followed by Scheffé's post hoc test. To determine the number of febrile phases, the data were plotted in the phase-plane format [rate of change of displacement vs. displacement; in our particular case, $T_c'(t)$ vs. T_c], and the number of loops (cycles) was then counted (16).

RESULTS

Experiment 1

At thermoneutrality the 10 μg/kg dose of LPS caused a T_c rise of 1.0–1.5°C in conscious, unstressed rats. The phase-plane plot clearly demonstrates the polyphasic (triphasic) pattern of this fever (Fig. 1). In a cool environment the same dose of LPS induced a different ($P < 0.003$) response, which was characterized by a smaller rise in T_c and a different T_c dynamic. The phase-plane plot showed, however, that the latter response was also triphasic (Fig. 2). The major difference between the two responses (i.e., at 30 and 22°C) was in the T_c dynamics during *phase I*. In thermoneutrality (Fig. 1), *phase I* resulted in an overall 0.4°C rise in T_c [on a phase plane, compare the abscissa at the start of *phase I* (20 min) with that at its end (74 min postinjection)]; $T_c'(t)$ was positive most of the time (the ordinate above 0). In contrast, the injection of LPS in a cool environment (Fig. 2) resulted in an overall T_c fall during *phase I* [compare the abscissa at the start of *phase I* (24 min) with that at its end (76 min postinjection)]; the $T_c'(t)$ after a transient rise became negative and remained below 0 for most of *phase I*.

Experiment 2

The increase in the dose of LPS changed the febrile response; the analysis of variance rejected the null hypothesis of the responses to 10, 100, and 1,000 μg/kg doses being the same ($P < 0.018$). The phase-plane

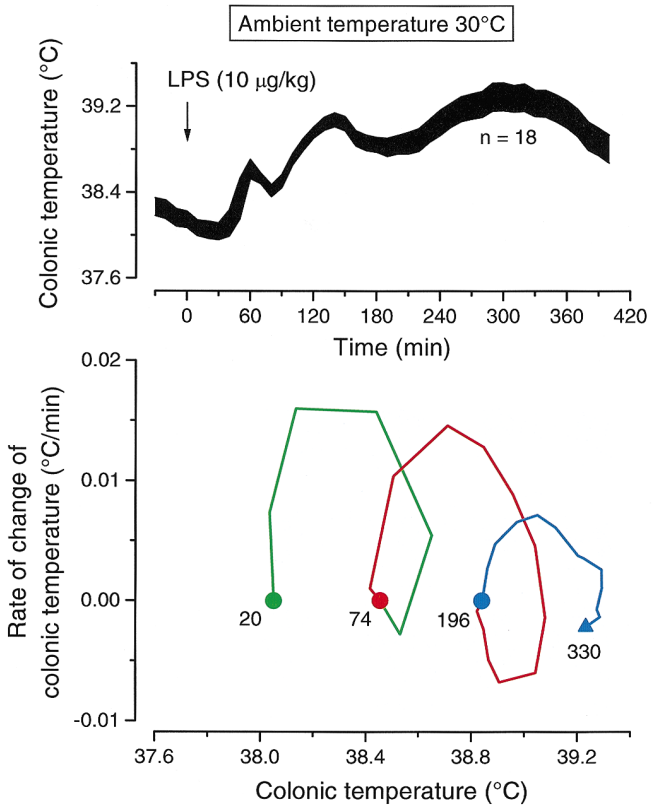

Fig. 1. Febrile response of rats to injection (arrow) of lipopolysaccharide (LPS; 10 μg/kg iv) at thermoneutrality shown as a time plot (*top*) and as a phase-plane plot (*bottom*). In phase plane, points marking beginning of each phase (chosen as points with 0 rate of T_c change and positive acceleration) are shown as ●; number near each data point corresponds to time elapsed after injection, in minutes. Three phases can be determined as 3 loops of curve (cycles): *cycle I* (green), from 1st circle (20 min postinjection) to 2nd circle (74 min); *cycle II* (red), from 2nd circle to 3rd circle (196 min); *cycle III* (blue), from 3rd circle to end of plot (▲, 330 min postinjection). For clarity, data points are plotted in phase plane at a 10-min interval, and dynamics of T_c during latent period of fever and during very end of experiment are not shown.

presentation (compare Figs. 1, 3, and 4) allows for the visualization of the effect of the increase in LPS dose on each febrile phase. *Phase I* changed most drastically: at 100 μg/kg, its maximal T_c and $T_c'(t)$ decreased two to three times (Fig. 3) compared with the response to 10 μg/kg (Fig. 1); as the dose was increased to 1,000 μg/kg (Fig. 4), *phase I* not only further decreased in magnitude but also resulted in a slight drop in T_c, rather than a rise. In contrast to *phase I*, *phase II* became more prominent as the dose increased: compare the distances between the starting point and the ending point of the second loop on Fig. 1 (~0.4°C), Fig. 3 (~0.9°C), and Fig. 4 (~1.1°C). *Phase III* did not change much with the increase of the dose from 10 to 100 μg/kg but became somewhat less pronounced at 1,000 μg/kg. Time graphs of an effector response (HLI) exhibit more obvious triphasic patterns than time plots of T_c (Figs. 1, 3, and 4) or ΔT_c (Fig. 5), especially for the case of higher LPS doses.

Experiment 3

Figure 6 shows how the febrile response of the rats to the 10 μg/kg dose of LPS depends on the injection method, i.e., with and without a needle prick. Without a needle prick, the response to LPS was triphasic, and none of these phases was due to the injection procedure per se (Fig. 6*A*). The T_c curve of the response to LPS of the needle-pricked rats was also triphasic and appeared similar to that of nonpricked animals ($P = 0.983$); however, *phase I* of this response was indistinguishable from the stress hyperthermia induced by the injection procedure per se (Fig. 6*B*). Therefore, in the case of the injection with a needle prick, it could have been easily concluded that the response to LPS was biphasic, occurring after the initial stress-associated (injection induced) hyperthermia.

DISCUSSION

Why Polyphasic Fevers Were Often Mistaken for Biphasic

The overall idea of the present study is that polyphasic fevers may easily be (and often are) mistaken for biphasic. Various factors can account for such a mistake, the most obvious of them being the length of the observation period. If, e.g., LPS fever is monitored in rats for 3 h postinjection only (19, 22), the response looks biphasic simply because no phase is recorded after *phase II*. This was the case in our recent study (17): biphasic fevers were reported (3 h postinjection), but the actual experimental records (when obtained for

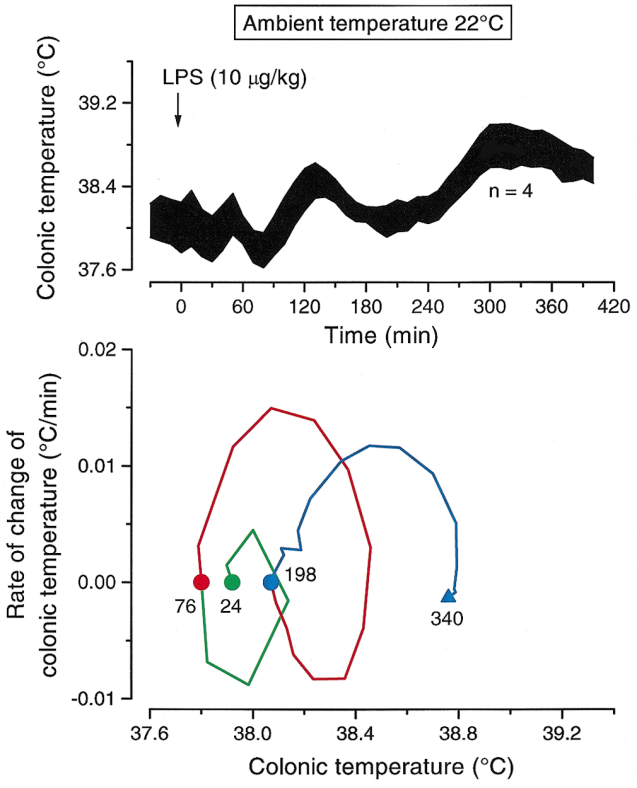

Fig. 2. Response of rats to injection (arrow) of LPS (10 μg/kg iv) in a slightly cool environment (*top*). In phase plane (*bottom*), 3 phases can be determined: *phase I* (green), from 1st circle (24 min postinjection) to 2nd circle (76 min); *phase II* (red), from 2nd circle to 3rd circle (198 min); *phase III* (blue), from 3rd circle to end of plot (▲, 340 min postinjection).

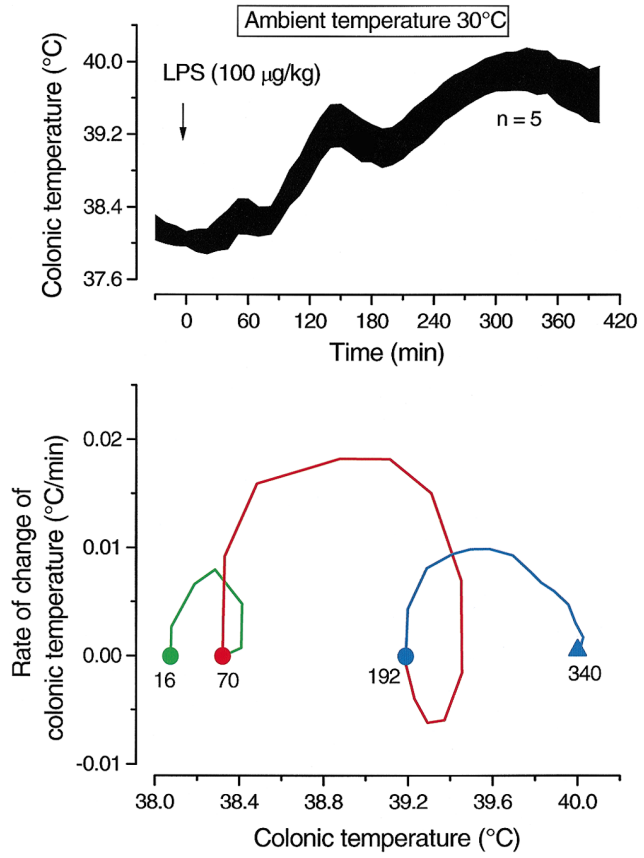

Fig. 3. Fever response of rats to injection (arrow) of LPS (100 μg/kg iv) at thermoneutrality (*top*). In phase plane (*bottom*), 3 phases can be determined: *phase I* (green), from 1st circle (16 min postinjection) to 2nd circle (70 min); *phase II* (red), from 2nd circle to 3rd circle (192 min); *phase III* (blue), from 3rd circle to end of plot (▲, 340 min postinjection).

a longer time) revealed polyphasic patterns indistinguishable from those seen in the present experiments.

Besides the observation time, other factors may interfere with the phase count. One of these factors is the T_a. The present study shows that if the T_a is neutral (30°C), the time plot of the LPS (10 μg/kg) fever demonstrates a clearly triphasic pattern; if, however, T_a is decreased to subneutral (22°C), the time plot becomes "confusing" (Fig. 2). Not surprisingly, therefore, responses similar to those shown in Fig. 2 are often described as "a slight decrease in T_c ... followed by a characteristic biphasic fever" (15). In the present work, by comparing the febrile response in a cool environment with that in a thermoneutral environment, we came to the conclusion that what resembles *phase I* at a subneutral T_a corresponds to *phase II* of fever at thermoneutrality and that what appears to be *phase II* is actually *phase III*. In other words, the pattern that is usually described as "hypothermia → *phase I* → *phase II*" is actually "hypothermia overlapping *phase I* → *phase II* → *phase III*."

The dependence of the pattern of the febrile response on the pyrogen dose also contributes to the difficulty of determining the "true" number of febrile phases. It has been emphasized (14, 17) that the higher the dose of LPS, the less pronounced *phase I* appears (although it starts earlier at higher doses) and the more prominent *phase II* becomes. The present study shows that at high doses febrile *phase I* almost completely vanishes and becomes practically unnoticeable in time plots (Fig. 5). Figure 5 also shows that the number of bursts of thermoeffector activity (waves of tail skin vasoconstriction, in our case) may be a more reliable indicator of the number of febrile phases than the T_b. Indeed, the T_b is more subject to inertia than thermoeffector activity: the activity of effectors directly changes the body's heat content and, therefore, is proportional to the rate of change of T_b, not to T_b per se. Then, if no thermoeffector is evaluated, a small T_b rise (resulting from a short-lasting burst of effector activity) is readily assumed to be statistically insignificant and disregarded as a febrile phase. Interestingly, the high "diagnostic value" of thermoeffector activity is compatible with the high diagnostic value of the phase plane, for which the rate of change of T_b [in our case, the $T_c'(t)$] is one of the two dimensions.

Finally, the present study identifies one more factor that is extremely important for correctly counting the number of phases: the method of pyrogen administration. Apparently, the same febrile response may be considered biphasic or polyphasic, depending on whether the pyrogen is administered with or without a

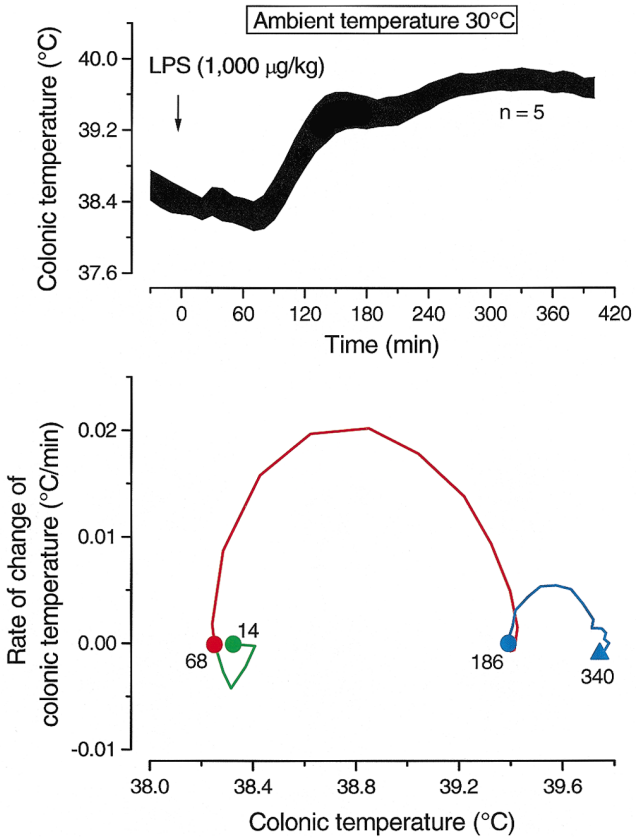

Fig. 4. Response of rats to injection (arrow) of LPS (1,000 μg/kg iv) at thermoneutrality (*top*). In phase plane (*bottom*), 3 phases can be determined: *phase I* (green), from 1st circle (14 min postinjection) to 2nd circle (68 min); *phase II* (red), from 2nd circle to 3rd circle (186 min); *phase III* (blue), from 3rd circle to end of plot (▲, 340 min postinjection).

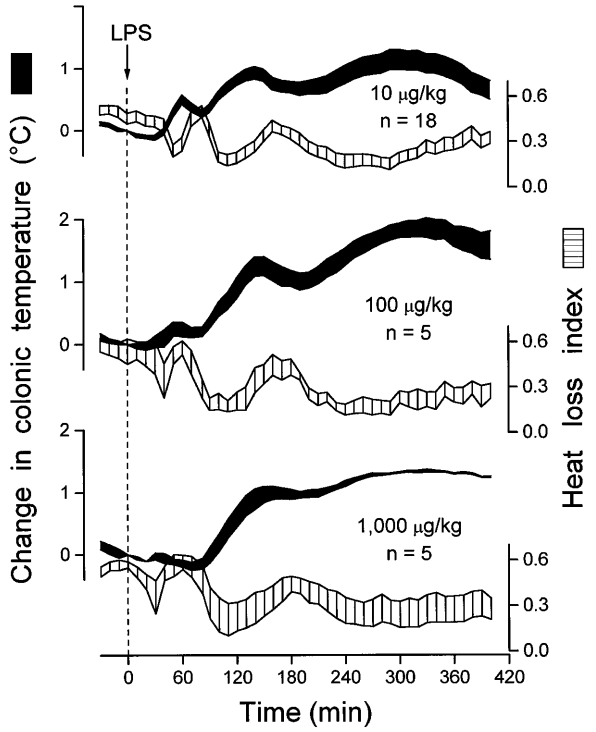

Fig. 5. Time plots of thermal (ΔT_c, left axes of ordinates) and effector [heat loss index (HLI), right ordinate axes] responses of rats to intravenous injection (arrow) of LPS at ambient temperature of 30.0°C. Upper border of filled and hatched areas, mean + SE; lower border, mean − SE. Plots of HLI clearly demonstrate triphasic pattern, whereas plots of ΔT_c do not.

needle prick. Figure 6 shows that, when injected without any needle prick, LPS induces the triphasic rise in T_b. Because the injection of PFS (also without a needle prick) has no effect on T_b, all three LPS-induced T_b rises are thought to be attributed to the action of LPS (not to the injection procedure), and the febrile response is considered truly triphasic. When, however, the injection is associated with a needle prick, PFS induces a rise in T_b (stress hyperthermia), which closely resembles febrile *phase I*. In this case, although the response to LPS consists of three consecutive T_b rises, the first rise is thought to be due to stress hyperthermia (has nothing to do with LPS per se); only the two remaining rises are regarded as febrile, and the fever response is thought to be biphasic (7, 9, 12, 18). As a complex combination of psychological and physical factors, the stress hyperthermia is characterized by great variability; the simultaneous development of the low-magnitude febrile *phase I* and the high-variability stress hyperthermia does not allow for the reliable detection of fever. Interestingly, the stress hyperthermia (sometimes called emotional fever) has been pointed out (2, 8, 10) as a potential explanation of the reported failures to induce fever in several vertebrate species.

An Important Corollary

In the recent literature on fever, the pyrogen is usually injected in one of two ways: intravenously (through a preimplanted catheter) or intraperitoneally (with the injection procedure involving animal handling and needle pricking). The two methods produce very different results: the febrile response of rats to the intravenous LPS (10–1,000 μg/kg; present paper) is characterized by a short latency (~10 min) followed by three T_b rises (peaks at ~1, 2.5, and 5 h postinjection), whereas the response to the intraperitoneal LPS (within the same dose range) (7, 9, 18) involves an ~90-min-long latent period (during which stress hyperthermia occurs; T_b peak at ~1 h) followed by two febrile phases (peaks at ~2.5 and 5 h). The difference between the two fevers is usually explained by the route of LPS administration (intravenous vs. intraperitoneal). The present results suggest that there may be another explanation. We hypothesize that the time course of the fever response is similar for the two routes but that the stress hyperthermia readily masks febrile *phase I* if the injection procedure per se involves a needle prick and animal handling. If this hypothesis is correct, results that are currently thought to be incomparable (obtained by different techniques) become highly comparable, but certain changes in our interpretation of the results are required. Our hypothesis could be tested directly: if, at thermoneutrality, a relatively low dose of LPS is injected intraperitoneally through a preimplanted catheter (in a way that the injection of the vehicle only produces no stress hyperthermia), the response should be similar in its dynamic to fevers described here and elsewhere (16, 17). Interestingly, in a recent study by Carlson (3), when a pyrogen was injected into freely moving rats intravenously or intraperitoneally (in both cases, through a preimplanted catheter), the responses of plasma hormones (ACTH

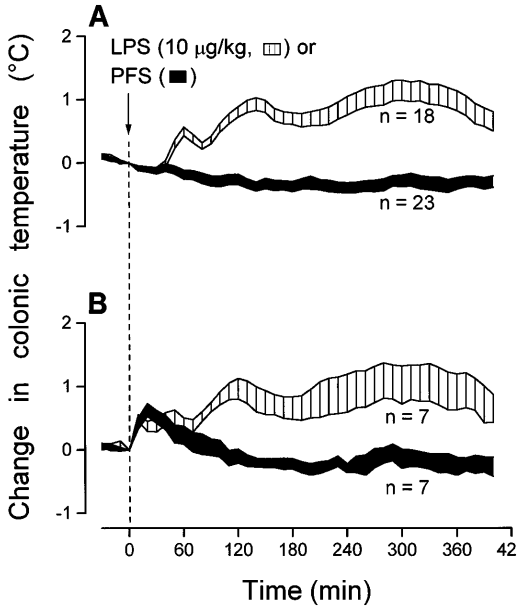

Fig. 6. Time plots of ΔT_c responses of rats to intravenous injection (arrow) of LPS (10 μg/kg) in pyrogen-free saline (PFS, 1 ml/kg) or PFS. In *A*, injection was through preimplanted jugular catheter, without disturbing animal, from outside experimental chamber. In *B*, same intravenous injection was also combined with a needle prick in lower abdomen. Ambient temperature is 30.0°C. Upper border of filled and hatched areas is mean + SE; lower border is mean − SE.

and corticosterone) showed the same dynamics, regardless of the route of administration.

Concluding Remarks

> Two live as one
> One live as two
> Two live as three
> Under the bam
> Under the boo
> Under the bamboo tree.
>
> (T. S. Eliot "Sweeney Agonistes")

The febrile response (at least the response of the rat to LPS) can have a triphasic (polyphasic) pattern. This pattern could be easily revealed if several methodological conditions are satisfied: the dose of LPS is not too high; the method of LPS administration does not involve stressing (handling and needle pricking) the animal; the experiments are run at thermoneutrality; T_b and thermoeffector activity are recorded; and the time of observation is ≥5–6 h postinjection. If any of these conditions is not satisfied, the number of phases can easily be miscounted due to one of the following reasons. If *phase I* is low in magnitude (the dose is too high and no effector activity is recorded) and/or if it is masked by the subsequent hypothermia (T_a below thermoneutrality), it can be easily overlooked; then, *phase II* is mistaken for *phase I*, *phase III* is mistaken for *phase II*, and the polyphasic pattern is mistakenly called biphasic. If *phase III* is missing because of insufficient observation time or simply is not obvious (too high a dose; no record of effector response), the response is described as biphasic. If all three phases are recorded, but *phase I* is assumed to be stress associated (handling and needle pricking during pyrogen administration), it is disregarded as a febrile phase; therefore, *phase II* becomes *phase I*, *phase III* becomes *phase II*, and the triphasic pattern becomes biphasic. Finally, in those early studies where several conditions identified here were not in effect simultaneously (e.g., T_a was low, the dose of LPS was high, administration of LPS was stressful, time of observation was short, and no effector activity was recorded), all phases of the febrile response were missed and conclusions such as "rats do not respond with fever to a single dose of ... endotoxin" (20) were typical. Thus the present study highlights once again that the outcome of a thermophysiological experiment strongly depends on its methodology (1).

Perspectives

Over the last decades, a great deal of research effort in the physiology of fever has been concentrated around the question of the differential triggering of the two febrile phases. Although this enormous effort has hardly resulted in any consensus (for review see Ref. 13), the two febrile phases are often viewed as two separate events, each having its own mediatory system. The present study, as well as our previous one (16), has shown that there are more than two febrile phases; does it mean that there are more than two systems mediating the febrile response? It may be so. Yet it is definitely plausible to suggest that a single trigger (whether represented by a single mediator or by a cascade of mediators) causes a febrile shift in T_b, which then oscillates at a new, elevated level (febrile *phases II, III,* and beyond), and that the development of these oscillations does not require the introduction of additional fever mediators. Although the literature contains some attempts to analyze oscillatory processes in the thermoregulatory system (4, 5), there is only one work directly aimed at testing the intriguing hypothesis of a single trigger for more than one febrile phase (13). That work, however, failed to demonstrate the oscillatory nature of febrile *phase II*, and additional research is required for more definite conclusions. Future studies on this topic may also include an analysis in the phase plane: although we have used the phase plane as a technique of topographic presentation of thermophysiological data (16), a formal analysis in the phase plane has yet to be performed.

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