Complement reduction impairs the febrile response of guinea pigs to endotoxin

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Sehic, E., S. Li, A. L. Ungar, and C. M. Blatteis. Complement reduction impairs the febrile response of guinea pigs to endotoxin. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1594–R1603, 1998.—Although it is generally believed that circulating exogenous pyrogens [e.g., lipopolysaccharides (LPS)] induce fever via the mediation of endogenous pyrogens (EP) such as cytokines, the first of these, tumor necrosis factor-α, is usually not detectable in blood until at least 30 min after intravenous administration of LPS, whereas the febrile rise begins within 15 min after its administration. Moreover, although abundant evidence indicates that circulating LPS is cleared primarily by liver macrophages [Kupffer cells (KC)], these do not secrete EP in immediate response. This would imply that other factors, presumably evoked earlier than EP, may mediate the onset of the febrile response to intravenous LPS. It is well known that blood-borne LPS very rapidly activates the intravascular complement (C) system, some components of which in turn stimulate the quick release into blood of various substances that have roles in the acute inflammatory reaction. KC contain receptors for C components and are in close contact with afferent vagal terminals in the liver; the involvement of hepatic vagal afferents in LPS-induced fever has recently been shown. In this study, we tested the hypothesis that the initiation of fever by intravenous LPS involves, sequentially, the C system and KC. To test this postulated mechanism, we measured directly the levels of prostaglandin E2 (PGE2) in the C system and KC. To test this postulated mechanism, we measured directly the levels of prostaglandin E2 (PGE2) in the intestinal fluid of the preoptic anterior hypothalamus (POA), the presumptive site of the fever-producing controller. Here, the cytokines are thought to stimulate the synthesis and release of prostaglandin E2 (PGE2), which is putatively considered to be the ultimate endogenous mediator of the febrile response. It is, however, controversial as to how cytokines could reach neurons in the POA because of the presence of a blood-brain barrier (BBB) that a priori precludes their free passage from the blood into the brain (reviewed in Ref. 4). Moreover, the appearance of any of these cytokines in blood lags by at least 15 min the onset of the rise in core temperature (Tc) after intravenous LPS administration, and both LPS and cytokines are per se weak triggers of the arachidonic acid cascade (4). The possibility that circulating pyrogenic signals may be transduced directly in the organum vasculosum laminae terminalis, which lies in the medial POA and lacks a BBB, or by POA microvessels generally, is similarly challenged by the fact that the kinetics of the various synthetic processes involved are slower than the latency of the febrile response to either intravenous LPS or cytokines (4). Hence, it may be speculated that an alternative mechanism of peripheral pyrogen signaling may operate. In view of the very quick onset of fever after the intravenous injection of LPS in particular, it may be further speculated that a neuronal rather than a humoral pathway may initially be involved.

Because LPS administered intravenously is cleared from the systemic circulation principally by the macrophages (Mφ) of the liver [Kupffer cells (KC)], and because the KC make up the single largest population of Mφ in the body, they are generally taken to be the major source of endogenous pyrogens induced by lipid A, the active principle of LPS (22, 28). Thus it is possible that the concentration of cytokines in the vicinity of their source of production may be sufficiently elevated earlier than in systemic blood and stimulate primary sensory afferents in the area. Indeed, it was recently reported that paraganglia on hepatic branches of the vagus bind interleukin-1 (IL-1) receptor antagonist (IL-1ra) (14), and other evidence was adduced that the vagus may provide a rapid communication pathway for cytokine signaling between the periphery and the brain (42). In support, we showed that subdiaphragmatic vagotomy inverted the Tc rise into falls and abrogated the concomitant increases in preoptic PGE2 levels induced in conscious guinea pigs by intravenous LPS (35). These data would thus suggest that fever may be initiated by cytokines released by activated KC into the microenvironment of the liver virtually immediately after LPS injection. However, although the

body temperature; Kupffer cells; gadolinium chloride; cobra venom factor; prostaglandin E2
binding of LPS to its receptors in Mφ occurs very rapidly, the induction by KC of tumor necrosis factor-α (TNF-α), the first of the cytokines to be produced, requires a minimum of 30 min of contact with LPS (21). Furthermore, because of their location in the hepatic sinusoids and continuous exposure to portal blood and, therefore, to gut-derived endotoxins, KC are less reactive (“tolerant”) to LPS, and their production of TNF-α and IL-1 is downregulated compared with Mφ elsewhere (5, 20). Hence, it may be considered that 1) the stimulus for KC cytokine production is not LPS per se but rather a secondary mediator occurring in almost immediate reaction to the presence of LPS in the blood, 2) the primary febrigenic target of LPS is not KC but other cell types, or 3) the neuroactive substance evoked by LPS and/or its mediator is not a cytokine but another factor presumably released earlier than TNF-α or IL-1.

The intravenous administration of LPS (a suspension of negatively charged macromolecular particles) triggers within 2 min the complement (C) cascade via both the classical and the alternative pathways by the reactions of the lipid A moiety of LPS with C1q and of core oligosaccharides with C3, respectively (reviewed in Ref. 41). This activation results in the production in blood of the anaphylatoxins C fragments C4a, C3a, and C5a. KC express their receptors, and it has been demonstrated in vitro that the KC production of cytokines is triggered after the addition of these fragments (6, 16). These anaphylatoxins also very rapidly stimulate the release into blood, by KC and other cells in the liver, of various noncytokine mediators that have demonstrated roles in the acute inflammatory reaction, e.g., PGE2 (30). We have therefore hypothesized that the initial step in the production of intravenous LPS–induced fever may involve, sequentially, C activation and KC stimulation by C components. To verify the presumptive, pivotal intermediary roles of C and KC in the early-phase induction of intravenous LPS fever, we depleted guinea pigs of C by using cobra venom factor (CVF) or temporarily eliminated the KC by pretreating the animals with gadolinium chloride and subsequently measured the animals’ Tc, and preoptic PGE2 levels after intravenous LPS. The results confirmed that an intact C system and KC contribute to developing a normal febrile response to intravenous LPS.

**METHODS**

Male Hartley guinea pigs (301–350 g; Sasco, St. Louis, MO) were used in these experiments. The animals were quarantined for 1 wk, three to a cage, before any experimental use. Tap water and food (Agway Prolab guinea pig diet) were available ad libitum. The ambient temperature in the animal room was 22 ± 1°C, and light and darkness were alternated, with light on from 0600 to 1800. After quarantine, to moderate the psychological stress associated with the experiments, the animals were trained to the experimental procedure for at least 5 days (daily for 3–4 h) by handling and placement in individual, locally fabricated, wire mesh stocks designed to prevent their turning around and to minimize their forward and backward movements but without causing restraint stress. The animals were anesthetized with ketamine-xylazine (50:5 mg/kg im) and prepared as described previously (36). Thus, briefly, immediately thereafter, a sterile guide cannula with an indwelling stylet was implanted stereotaxically into the left medial POA of each guinea pig [coordinates relative to the interaural axis (in mm): AP 11.6, L 1.0, V 8.5] and fixed to the skull with four self-tapping, miniature stainless steel screws and dental acrylic cement. Four days later, a siliconized cannula prefilled with heparinized (10 U/ml) pyrogen-free saline (PFS) was inserted into a jugular vein of each guinea pig and exteriorized at the top of the head. It was flushed with heparinized PFS (3 U/ml) daily; 48 h before an experiment, heparinized PFS was replaced with PFS only (43). Three days before an experiment, the indwelling stylets in the preimplanted guide cannulas were replaced by microdialysis probes such that the dialysis membrane tips protruded 1.5 mm beyond the guides. All the animals received gentamicin sulfate (6 mg/kg im) prophylactically before each surgical procedure.

Three days after the last surgery, the guinea pigs, fully conscious, were loosely restrained in individual wire mesh cages at an ambient temperature of 23.0 ± 1.0°C. The Tc of the guinea pigs were monitored constantly and recorded at 2-min intervals for the duration of the experiments on an Apple IIe microcomputer through an analog-to-digital converter using a calibrated copper-constantan thermocouple inserted 5 cm into the colon. The data were displayed graphically on a monitor, printed digitally on an Imagemaker printer, and stored on a floppy disk. The effluents from the microdialysate probes were collected over 30-min periods continuously throughout the experiments, and the samples were analyzed by radioimmunoassay for their PGE2 content.

A 90-min stabilization period to achieve thermal equilibrium preceded all the treatments. To obviate possible effects of circadian variations, the experiments were begun at the same time of day (0830). The following experiments were conducted.

**Experiment 1.** Artificial cerebrospinal fluid (aCSF) was microdialyzed into the POA of guinea pigs at the rate of 2 µl/min. Ninety minutes after the beginning of microdialysis, a bolus injection of 0.2 ml PFS or 2 µg LPS/kg body wt in 0.2 ml PFS was made through the preinserted venous cannula over a 10-s period. The microdialysis continued uninterruptedly for an additional 4 h.

**Experiment 2.** Guinea pigs were injected through their intravenous cannulas with PFS or CVF at 200 U/animal, delivered according to the following schedule: 50 U initially at 1230, 50 U 2 h later, and 100 U 18 h after the second dose (0830 of the following day); the animals remained free in their home cages during all but the last 90 min of this period. This paradigm was adopted on the basis of preliminary dose–response and duration–of–effect studies designed to determine the conditions that would achieve maximal, sustained reductions in serum C levels (3). The administration of CVF was staggered to minimize untoward reactions that sometimes occurred in our preliminary studies when the larger doses of CVF were delivered in single injections. This schedule also minimized interference with our habitual experimental protocols. Thus 1 h after the last CVF dose, at 0930, the microdialysis of aCSF into the POA was begun and, 90 min later, a bolus injection of 0.2 ml PFS or 2 µg of LPS/kg body wt was made through the preinserted venous cannula. A separate study was also conducted to evaluate the effect of this treatment schedule on the Tc of conscious guinea pigs; in this case, the animals were restrained during the first 5.5 h and then again during the last 4 h of the 21-h pre-PFS or pre-LPS period (see Fig. 2). Immediately before and 21 and 25 h (i.e., the latter at the conclusion of the experiment) after the initial administra-
tion of PFS or CVF, blood (200 µl) was withdrawn through the cannula for measurement of serum total hemolytic C activity (expressed as CH100 units) by a radial diffusion method. Briefly, 5 µl of serum samples were added to wells placed in agarose gel containing standardized sheep erythrocytes sensitized with hemolysin (kit no. RC001; The Binding Site, San Diego, CA). Plates were incubated for 18 h at 4°C and then for 1 h at 37°C. The areas of the zones of hemolysis around each well were measured by imaging these zones with a charge-coupled device camera then scanning the images and calculating their relative optical densities using the National Institutes of Health Image version 1.61 for analyzing electrophoretic gels. These values were converted to CH100 units by interpolation from calibration curves plotted using the manufacturer’s standard, diluted from neat to 1:32 (minimum sensitivity, 32 CH100 units).

Experiment 3. A separate group of animals with preinserted venous cannulas received a single injection of gadolinium chloride hexahydrate (GdCl3; 7.5 mg/kg). Three days after GdCl3 pretreatment, aCSF was microdialyzed into the POA at the rate of 2 µl/min for 5.5 h. Ninety minutes after the beginning of microdialysis, a bolus injection of 0.2 ml PFS or 2 µg of LPS/kg body wt in 0.2 ml PFS was made through the preinserted venous cannula, as before.

Experiment 4. To preclude the possibility that any GdCl3 that might have penetrated the brain could impair its capacity to produce PGE2, an a posteriori experiment was conducted in which guinea pigs were prepared exactly as in experiment 3 except that 90 min after the beginning of microdialysis, in lieu of the intravenous injection of PFS or LPS, the aCSF perfusate was changed to aCSF containing 10 µg of norepinephrine (NE) or its vehicle per microliter of aCSF, buffered to pH 7.4 with 1 N NaOH and prepared just before use; under these conditions, NE directly stimulates the release of PGE2 in the POA (36).

After an experiment, the animals were anesthetized through their intravenous cannulas and decapitated with a guillotine. Their brains were removed and stored in 10% phosphate-buffered Formalin for biological verification of the placement of the dialysis probe tips. Only the data from guinea pigs with confirmed preoptic placement of the probes are included in this report.

Drugs. The aCSF microdialysis perfusate was prepared as follows (final concentration, in mM): 140.0 NaCl, 2.7 KCl, 1.0 MgCl2 · 2H2O, 1.2 CaCl2 · 2H2O, and 2.0 Na2HPO4; osmolality, 290 mosmol/kgH2O; pH 7.4, adjusted with 85% H3PO4. The vehicle for all intravenous injections was PFS (0.9% NaCl, USP; Abbott Laboratories, Chicago, IL). Heparin was purchased (no. NEK-020A) from DuPont (Wilmington, DE); its detection limit was 0.25–1.00 pg/100 µl in experiments 1 and 3 and 1–10 pg/100 µl in experiments 2 and 4, due to a change in the PGE2 antibody component of the manufacturer’s kit in the intervening time. CVF (Naja naja kaouthia) was purchased from Calbiochem-Novabiochem (San Diego, CA), and GdCl3 hexahydrate was purchased from Aldrich (Milwaukee, WI). LPS was Salmonella enteritidis LPS B (batch no. 651628; Difco Laboratories, Detroit, MI) suspended in PFS. [-]-NE (10 µg) was its bitartrate salt, along with 2 µg sodium metabisulfite (Sigma, St. Louis, MO) per microliter aCSF; its vehicle was 5 µg sodium hydrogen tartrate (Aldrich) and 2 µg sodium metabisulfite per microliter aCSF.

Statistical analyses. The results are reported here as means ± SE. The values of TC are the changes from basal values (Tc = the Tc at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period) plotted at 6-min intervals. The PGE2 data are expressed as percent changes relative to their last value before a treatment (P3), to minimize the individual variations in basal PGE2 levels among animals. Student’s paired t-test was used to compare pretreatment (basal) and posttreatment (maximal) data within a treatment. Differences between treatments were evaluated by a repeated-measures analysis of variance model, in which factor 1 was the between-groups factor (the experimental treatment) and factor 2 the within-subject factor (the different sampling periods). Each variable was considered to be independent. The 5% level of probability was accepted as statistically significant.

RESULTS

Experiment 1. Continuous unilateral microdialysis of aCSF into the POA for 5.5 h had no effect on the Tc, or on the PGE2 levels in the microdialysate effluents from the POA of the guinea pigs given intravenous PFS (Fig. 1). The animals that received intravenous LPS, by contrast, exhibited characteristically biphasic, ~1.4°C in-
creases in their Tc, as well as two- to sixfold increases in their mean preoptic PGE2 levels, in apparently close correlation with their febrile course (Fig. 1).

Experiment 2. CVF at 200 U reduced the total hemolytic C activity of serum from 1,036.6 ± 76.6 and 1,066.7 ± 59.4 CH100 units before CVF to 43.2 ± 4.5 and 42.0 ± 11.2 CH100 units (a 96% reduction) just before PFS (n = 7) and LPS (n = 8) administration, respectively, 21 h later. At the conclusion of the experiments, these values were 60.3 ± 25.1 and 52.8 ± 9.8 CH100 units, respectively. LPS per se caused a 12% reduction in CH100 within 10 min (P < 0.05), which was restored after 6 h. PFS pretreatment had no demonstrable effect on C activity in the corresponding control animals. The initial 50-U dose of CVF rapidly induced a transient, ~1°C decrease in the animals’ Tcs. The fall began within 10 min after the injection, reached its lowest level in 45 min, and then, after another 15 min, gradually returned toward its original value during the following 45 min (Fig. 2). But neither the second injection of 50 U of CVF 2 h after the first nor the third of 100 U 18 h after the second had any effect on Tc. Hence, 21–25 h after the initial dose of CVF, the Tc and preoptic PGE2 levels of the decomplemented guinea pigs (Fig. 3) were not demonstrably affected by the CVF treatment compared with their PFS-treated, C-sufficient controls (Fig. 3). The subsequent intravenous injection of PFS (Fig. 4) also had no effect on their Tc and POA PGE2 levels, whereas that of LPS (Fig. 4) resulted in a significantly attenuated first rise of the two rises of Tc characteristically evoked by this dose of LPS (Fig. 1) and the inversion of the second rise into an ~1.0°C fall. The normally fever-associated increase in POA PGE2 levels also was suppressed in these C-depleted animals.

Experiment 3. Intravenous PFS caused no change in Tc, or POA PGE2 levels of the GdCl3-pretreated animals (Fig. 5), whereas intravenous LPS (Fig. 5) induced an ~1.0°C fall in Tc but did not significantly affect preoptic PGE2 levels. Twenty-five days after GdCl3, however, these animals were again able to evoke prototypic febrile responses to LPS (delivered in this instance at 10 μg/kg im because, in the meantime, the intravenous cannulas were no longer patent) (Fig. 6). The guinea pigs gained ~150 g in body weight over this interval.

Experiment 4. The microdialysis of buffered NE into the POA of guinea pigs treated 3 days previously with GdCl3 caused a prompt and significant elevation of their preoptic PGE2 levels. The increase was evident within the first 30 min of the perfusion, became greater over the following two to three collection periods, then stabilized at ~200% above its control (P3) level for the duration of the perfusion (Fig. 7, bottom). The vehicle of NE had no effect on the preoptic PGE2 levels of GdCl3-pretreated guinea pigs (Fig. 7). Also, neither NE nor its vehicle significantly affected the Tc of these guinea pigs (Fig. 7, top).

DISCUSSION

The present results indicate that guinea pigs rendered hypocomplementemic by the prior intravenous injection of CVF develop a considerably reduced first rise and a fall rather than a second rise in Tc, and fail to exhibit an increase in preoptic PGE2 levels in response to the subsequent intravenous administration of a pyrogenic dose of LPS that normally induces a biphasic
fever. CVF by itself causes an initial, transient fall in $T_c$ but no further, evident thermal response when readministered 2 and 18 h after the original dose; preoptic PGE$_2$ levels are not different from their untreated controls at 21 h. Furthermore, guinea pigs pretreated with GdCl$_3$, a lanthanide that temporarily eliminates Mφ, in particular KC within the time frame of this study (15), similarly are unable to develop febrile and preoptic PGE$_2$ responses to intravenous LPS. GdCl$_3$ by itself has no thermal or PGE$_2$ effect when assessed 3 days later, nor does it impede the capacity of the POA to generate PGE$_2$ when stimulated directly by NE. These observations suggest, therefore, an integral association of the C system and of KC with intravenous LPS-induced fever and the production of its putative central mediator, PGE$_2$.

It is generally believed that fever caused by LPS administration is mediated by the LPS-induced release of pyrogenic cytokines into the circulation and their transport to the brain by the bloodstream. Indeed, elevated plasma levels of TNF-$\alpha$, IL-1, and IL-6 have variously been reported after intravenous LPS. However, although the active transport of these cytokines into the brain of rats and mice has been shown (2), the amount transferred is <1% of their concentration in blood, and the process is significantly delayed compared with fever onset latency. The possibility that they may pass, or their messages be transduced in, circumventricular organs in which the BBB is leaky, specifically in the organum vasculosum laminae terminalis, or in endothelial cells of the POA vasculature, is also challenged by the fact that LPS-induced cytokines generally are not detectable in blood until after the onset of fever (4). On the other hand, their concentration may be expected to be elevated earlier in the vicinity of their sources of production than in systemic

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Fig. 4. Effects of iv-injected PFS or LPS on $T_c$ (top) and interstitial fluid levels of PGE$_2$ (bottom) in POA of conscious, CVF-pretreated guinea pigs with unilaterally implanted microdialysis probes, evaluated 21–25 h after initial dose of CVF. Conventions as in Fig. 1.

Fig. 5. Effects of iv-injected PFS or LPS on $T_c$ (top) and interstitial fluid levels of PGE$_2$ (bottom) in POA of conscious, gadolinium chloride (GdCl$_3$)-pretreated (3 days prior) guinea pigs with unilaterally implanted microdialysis probes. Conventions as in Fig. 1.

Fig. 6. Effect of im-injected PFS or LPS on $T_c$ of conscious, GdCl$_3$-pretreated (25 days prior) guinea pigs. Conventions as in Fig. 1.
Blood, and, to account for the rapid and concomitant increases in \( T_c \) and preoptic \( \text{PGE}_2 \) levels after intravenous LPS, a neural signaling pathway may be presumed to exist between these production sites and the brain. Although the phagocytes lining the pulmonary artery constitute the first filter encountered by LPS injected intravenously, the rate of LPS clearance and detoxification by these cells is very slow (28), thereby allowing the spillover of LPS into the general circulation. Consequently, monocytes and other \( \text{M} \phi \) resident within the vasculature, i.e., hepatic and splenic, also contribute to the intravascular clearance of LPS. Of these, \( \text{KC} \) are quantitatively the most important (\( \text{KC} \) constitute 80% of all resident mononuclear phagocytes in the body). The liver is therefore generally considered to be the principal organ responsible for clearing LPS from the blood (22). Indeed, as pointed out earlier, evidence was recently adduced that subdiaphragmatic vagotomy suppresses the febrile responses to peripheral LPS and IL-1 and that hepatic vagal branch-associated paraganglia bind IL-1ra, suggesting that the pyrogenic message may be transmitted centrally by activated vagal afferents originating within the liver (14, 35).

Although the involvement of \( \text{KC} \) in LPS uptake and the capacity of \( \text{KC} \) to produce cytokines are well documented, relatively little is known about their precise role in the in vivo production of LPS-induced fever. We hypothesized that, if \( \text{KC} \) were indeed the major source of increased cytokine bioactivity after intravenous LPS, their elimination should prevent cytokine release and, hence, fever, thus confirming the critical role of these cells in fever production. To test this proposition, we used \( \text{GdCl}_3 \), a lanthanide rare earth metal that inactivates \( \text{M} \phi \) within the vasculature, i.e., hepatic, splenic, and pulmonary intravascular \( \text{M} \phi \), \( \text{Gd}^{3+} \) has a similar ionic radius as \( \text{Ca}^{2+} \) and a higher charge density; it thus competes avidly for \( \text{Ca}^{2+} \) binding sites on both low- and high-threshold channels, inhibiting \( \text{Ca}^{2+} \) influx and signal transduction, eventually leading to \( \text{M} \phi \) apoptosis (26). Because of their higher phagocytic and lysosomal activities, the larger \( \text{KC} \) in the periportal zone of liver acini are affected significantly within 12 h after treatment, whereas the smaller, less efficient, midzonal and perivenous \( \text{KC} \) and splenic and pulmonary intravascular \( \text{M} \phi \) are less vulnerable (19, 38). Repopulation of splenic and pulmonary \( \text{M} \phi \) reportedly starts at 1 day and of \( \text{KC} \) at 4 days after \( \text{GdCl}_3 \) injection (19). In the interval, phagocytosis by the larger \( \text{KC} \) is reduced 70–80%, but that by the smaller \( \text{KC} \) and by splenic \( \text{M} \phi \) is increased compensatorily (19, 31); \( \text{GdCl}_3 \) has no demonstrable effect on endothelial and parenchymal cells (19). We too have detected FITC-labeled LPS within the liver sinusoids of control and \( \text{GdCl}_3 \)-pretreated conscious guinea pigs 15 min after its intravenous administration (37). In this study, we therefore administered LPS 3 days after \( \text{GdCl}_3 \) pretreatment, when the larger periportal \( \text{KC} \) had presumably been functionally eliminated from the liver, whereas the smaller ones were extant and all other \( \text{M} \phi \) were reappearing. Because the intravenous injection of LPS under these conditions did not induce rises in either \( T_c \) or preoptic \( \text{PGE}_2 \) levels, it may be inferred that the presence of the larger \( \text{KC} \) in particular is critical for the induction of the febrile response to intravenous LPS. By the same token, assuming that, in consequence of the reduction in the larger \( \text{KC} \), the phagocytosis of LPS, like that of other particulate substances, by smaller \( \text{KC} \) and by splenic \( \text{M} \phi \) is increased compensatorily, it may be further inferred that their thus enhanced sensitivity is nevertheless insufficient to stimulate their rapid production of pyrogenic mediators, at least under the present experimental conditions. Moreover, it is noteworthy that, despite the elimination of the large \( \text{KC} \), \( \text{GdCl}_3 \) pretreatment is reported to lead to significantly increased liver levels of TNF-\( \alpha \) transcripts (31); i.e., their source is \( \text{GdCl}_3 \)-insensitive cells. Nonphagocytic cell types in liver that produce TNF-\( \alpha \) as well as IL-1 and IL-6 in response to LPS are hepatocytes and endothelial, biliary epithelial, and mast cells; their contribution, however, does not lead to significantly increased cytokine levels in blood (33). Hence, it would seem that LPS activation of these cells is also not relevant to the initiation of fever under the present experimental conditions. The possibility that \( \text{GdCl}_3 \) per se might have exerted a direct, inhibitory effect on the POA's...
ability to produce PGE\(_2\) is negated by the present demonstration that, in conformity with our earlier findings (36), the POA of GdGl3-pretreated guinea pigs releases PGE\(_2\) normally in response to locally microdi-
alyzed NE. The lack of an accompanying Tc rise in this instance is due to the presence of antioxidants in the perfusion medium (36). To our best knowledge, this is the first demonstration that the presumptively selective elimination of large KC suppresses the febrile response to intravenous LPS, although earlier studies using different compounds, in particular liposome-encapsulated drugs, have also shown that the depletion of peripheral M\(_\phi\) generally in organs with an open circulatory system results in various, altered, LPS-induced host defense responses (9). As is characteristic of most experimental manipulations that attenuate host defense responses (36), the POA of GdGl3-pretreated guinea pigs are still unable to secrete its biologically active, mature forms, the expression of C3b and iC3b receptors (CR1 and CR3) on neutrophils was significantly augmented (27); this enhanced expression is normally induced by C5a (11). Taken together, these observations would suggest that the C system may have a proximal role in the pathogenesis of fever. Examples of lost functions that normally are coactivated with fever include the inability of C-deficient animals to enhance phagocytosis and promote leukocytosis in response to infection and to release reactive oxygen metabolites, eicosanoids, cytokines, chemotactic factors, lysosomal enzymes, and other inflammatory mediators (reviewed in Ref. 40).

The preceding notwithstanding, it should be noted that clearance of circulating LPS does not distinguish between adherence and phagocytic uptake and processing and also does not equate temporally with intracellular signaling for, e.g., cytokine release. Thus, although we (37) and others (25) have localized LPS on KC by fluorescent labeling within 15 min after its intravenous injection, at least 30 min of contact with LPS are required for maximal release of TNF-\(\alpha\) (21). As already noted, this interval is longer than the latency of fever onset after LPS intravenous injection. Similarly, although 5–10 min of LPS exposure is sufficient to trigger maximal TNF-\(\alpha\) release (4 h later) by human monocytes, little TNF-\(\alpha\) appears in blood (13). Moreover, although KC synthesize IL-1 mRNA in vitro within 30 min after LPS treatment, they apparently are unable to secrete its biologically active, mature form (1). In addition, in vivo, as already mentioned, KC are evidently less reactive than other M\(_\phi\) to LPS by virtue of their continuous, low-grade exposure to intestine-derived LPS, which contributes to the development of early-phase tolerance to its effects (5) and thereby prevents systemic sensitization by LPS. In sum, it would seem unlikely that KC-generated cytokines could be the mediators that rapidly trigger fever in response to an intravenous bolus of LPS. Other sources or more quickly evoked mediators may thus be presumed to be involved in initiating the febrile response to intravenous LPS.

The involvement of C in LPS-induced host defense responses is well documented. Thus it is long established that LPS activates the classical and alternative C pathways (41) and that by-products of the activation sequence released into the blood, in particular the anaphylatoxins, C4a, C3a, and C5a, and the membrane attack complex (MAC), C5b-9, are important humoral mediators of certain features of the generalized systemic response. Indeed, C activation is associated with the consumption of these components so that a reduc-
in their blood concentrations is observed in many inflammatory diseases (32). The importance of C in host defense is further attested by the susceptibility of congenitally C-deficient and decomplemented animals to recurrent infections with a wide variety of organisms and to autoimmune diseases. However, few studies have examined the possible role of C in fever production. Mickenberg et al. (23, 24) reported that total hemolytic C activity and C3 titers of rabbits fell within 5 min after the intravenous administration of low-dose, soluble antigen-antibody (AG-AB) complexes, in correlation with an attenuated febrile course. Furthermore, rabbits depleted of C by pretreatment with CVF exhibited diminished febrile responses in comparison with untreated controls. On the other hand, no anaphyla-
toxic C fragments were detected in the plasma of human subjects injected intravenously with low doses of Escherichia coli LPS, although Tc and plasma TNF-\(\alpha\) and IL-6 levels rose after 30–45 min (39). But importantly, despite the apparent absence of plasma C components, the expression of C3b and IC3b receptors (CR1 and CR3) on neutrophils was significantly augmented (27); this enhanced expression is normally induced by C5a (11). Taken together, these observations would suggest that the C system may have a proximal role in the pathogenesis of fever. Examples of lost functions that normally are coactivated with fever include the inability of C-deficient animals to enhance phagocytosis and promote leukocytosis in response to infection and to release reactive oxygen metabolites, eicosanoids, cytokines, chemotactic factors, lysosomal enzymes, and other inflammatory mediators (reviewed in Ref. 40).

Therefore, to test the hypothesis that C activation is integral to LPS fever production, we hypocomplement-
mented guinea pigs with CVF and measured their febrile and preoptic PGE\(_2\) responses to intravenous LPS. CVF activates the alternative pathway of the C cascade; it acts similarly to C3b, i.e., it forms a bimolecu-
al complex with factor B, the C3/C5 convertase CVF Bb (8). The function of CVF Bb is analogous to that of the natural C3b Bb, that is, to cleave catalytically the \(\alpha\)-chains of C3 and C5. Whereas C3b Bb is very labile [time to 50% disappearance (t\(_{1/2}\)) = 1.5 min at 37°C], CVF Bb is rather stable (t\(_{1/2}\) = 7 h). Furthermore, C3b Bb is regulated by factors H and I, whereas CVF Bb is resistant. Consequently, CVF leads to continuous, unregulated fluid-phase C activation, resulting in the depletion of C3 and all the subsequent C components, while sparing C1, C4, and C2. In this way, the further generation of the late components of the C cascade is markedly reduced because of the depletion of the substrates from which they are produced, leading to hypo-complementemia. CVF is a far more potent activator of C3 than LPS. Hence, in the present study, the finding that fever did not develop in response to intravenous LPS after CVF administration would indicate that an intact C system, and more specifically the alternative pathway, is required for LPS fever to de-
velop. The fact that no Tc rise developed after CVF would also suggest that activation of the alternative pathway per se does not contribute directly to fever
production. Indeed, $T_c$ fell rather than rose after the first 50-U dose of intravenous CVF. It is probable that this initial, transient $T_c$ fall was due to the anaphylatoxin-mediated release by mast cells and basophils of histamine and other vascular relaxing factors, thereby promoting vasodilation, including that of the cutaneous circulation. The absence of any further thermal effect of CVF was likely due to the significant reduction of C levels (−80%) caused by the first dose (3); i.e., CVF was introduced into a system exhausted of its precursor materials and, hence, also of the effector products. It cannot be determined from the present data which missing individual C component may be responsible for the impaired febrile response to LPS, but, because a major role of C in the effector phase of host defense is to mark foreign materials with C3 fragments and target them to various effector cells having C receptors, it is possible that C3 may promote the febrile response in concert with LPS, e.g., by LPS particles opsonized by iC3b and recognized by KC (44). Like KC depletion, C depletion resulted in the depression of the first $T_c$ rise and the inversion of the second rise into a fall. Again, the relationship of this pattern to the role of C is elusive, but it is possible that, in the present instance, the fall in $T_c$ caused by LPS in the CVF-treated animals was due to the unrestrained activation of directly evoked secondary mediators, e.g., histamine, cyclooxygenase products, and platelet activating factor, strongly exerting their systemic vasodilatory activity. To our best knowledge, this is the first demonstration that C, and the alternative pathway in particular, may play a pivotal role in the induction of the febrile response to, specifically, intravenously administered LPS. It is significant in this conjunction that the intravenous injection of LPS also induced under the present experimental conditions the transient, small, but significant consumption of C. It should be emphasized at this point, however, that this proposed involvement of C applies so far only to LPS-induced and AG-AB-induced (23, 24), but not other, fevers. Indeed, the pathogenesis of clinical infections is not constantly endotoxin related, some gram-negative and all gram-positive bacteria are not susceptible to C-mediated killing, and congenitally C-deficient, clinically infected patients can present with fever (29), albeit syndromes in which this latter feature is specifically mentioned are few.

Although the anaphylatoxins and MAC are independently capable of inducing cytokine production by Mφ, including KC, it is generally assumed that LPS interacts with Mφ to generate cytokines without a role for C, for it is certainly possible to activate Mφ in a C-independent way. However, LPS is inefficient at triggering, e.g., IL-1 mRNA from Mφ cultured in serum-free conditions at concentrations <2 ng/ml, and it has been shown that the response can be greatly amplified by tandem LPS and C activation; i.e., Mφ bearing the requisite C receptors receive signals from C fragments that synergize with the direct effect of the binding of LPS-lipoprotein binding protein to plasma membrane-bound CD14 receptors on the surface of Mφ to enhance the production of cytokines (44); another phagocyte receptor, CD11c/CD18 (CR4), may similarly bind LPS and activate cells (16, 17). However, the time course of synthesis of bioactive cytokines induced by these combinations of C and LPS in vitro nevertheless lags the latency of fever onset after LPS intravenously in vivo, and, although the transcription of IL-1 and TNF-α after C activation may be a little quicker than after LPS activation, it does not progress to translation and secretion (7, 34). Hence, in view of the evidently critical importance of KC in LPS fever genesis demonstrated in this study, it is logically conceivable that noncytokine rather than cytokine mediators released by these cells almost immediately after C activation could provide the initial signal for fever onset.

Thus, because the guinea pigs treated with CVF to deplete C3 and its sequences and with GdCl3 to eliminate KC were rendered less susceptible to fever by intravenous LPS than untreated animals, the implication is that the induction of the febrile response does not occur in the absence of C reactions at the surface of KC and the consequent, rapid generation by these cells of, probably, noncytokine products. Indeed, in addition to cytokines, the split products of C3, i.e., C3a and C3b, dose-dependently elicit the release of indomethacin-sensitive arachidonic acid metabolites from Mφ. For example, C3a stimulates PGE2 production by KC within 2 min (30), i.e., a time course that precedes the LPS-induced increase in Tc; C5a and its desArg form are also potent stimuli of PGE2 synthesis. Pretreatment of rabbits and pigs with C-depleting agents (CVF, methylprednisolone, and rosmarinic acid) prevents the rise in plasma prostaglandins induced by LPS (12). Furthermore, the addition of indomethacin results in detectable IL-1 activity in supernatants of Mφ cultures stimulated with suboptimal amounts of C3a desArg (7), whereas PGE2 inhibits the LPS-induced synthesis of IL-1 and TNF-α (but not IL-6) in KC (18), inferring again that these cytokines cannot account directly for the rapid febrile response to intravenous LPS. Hence, these results would suggest that the fever mediator released by KC could be PGE2, triggered by LPS-bound activated C3a and/or C5a. The thus secreted PGE2 could then bind to relevant receptors in vagal afferents distributed in the liver acini and/or in the nodose ganglion (10). Alternatively, the rapid uptake and degradation of KC-derived PGE2 by hepatocytes, which carry receptors for PGE2, may induce the consequent release of other, as yet unknown mediators capable of activating vagal afferents. Other parenchymal liver cells responsive to PGE2 may be similarly stimulated to release such mediators. It is also possible that C fragments could directly induce these mediators independently of PGE2. Finally, local effects of CVF or GdCl3 with systemic consequences, or systemic effects of CVF and GdCl3 on Mφ generally, but not tied to normal, physiological, febrigenic mechanisms, cannot be discounted. However, all these are speculations still in need of experimental verification.

In summary, we conclude that C activation and KC are necessary for the full development of the intravenous LPS-triggered febrile response in guinea pigs, and
we suggest that the rapid onset of this fever may occur independently of blood-borne cytokines. Furthermore, the close temporal and quantitative correlations between the depressed rises in Tc and preoptic PGE2 levels after intravenous LPS of the CVF- and GdCl3 pretreated guinea pigs are consistent with the hypothesis that mediator(s) released by C-activated KC may stimulate nearby vagal afferents that transmit febrigenic signals ultimately to the POA (4).

Perspectives

It is generally considered that the pathogenesis of fever involves sequential actions by exogenous pyrogens (such as LPS), endogenous pyrogens (represented by cytokines), and PGE2. The present results would suggest, however, that the febrigenic process may be more intricate than envisaged. Indeed, increasing evidence is accumulating from various studies that diverse mechanisms may underlie the febrile response. Thus distinct potencies and response profiles have been demonstrated among different pyrogen types, routes of administration, and test species. Hence, it is plausible that the apparently crucial importance of the KC in the febrile response to intravenous LPS uncovered in this study may be irrelevant in the case of LPS that infiltrates the lungs or the peritoneum and does not spill over into the systemic circulation. Similarly, exogenous pyrogens that do not inherently activate the C cascade may exert their febrigenic action perfectly competently in C-deficient hosts. Furthermore, it seems probable that the mechanisms that initiate and those that maintain fever may be different. Because, in addition, an array of secondarily evoked, peripheral and central agents, both facilitatory and inhibitory, also have roles, albeit as yet not well delineated, in the modulation of the febrile response at different stages of its course, the challenge becomes the unravelling of the network of interactions, among the manifold mediators, from peripheral exogenous pyrogen to the fever-producing site in the POA. The improved understanding of this febrile process may lead to better strategies for effectively controlling infectious disease processes in general.

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