Relation between complement and the febrile response of guinea pigs to systemic endotoxin

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Li, S., E. Sehic, Y. Wang, A. L. Ungar, and C. M. Blatteis. Relation between complement and the febrile response of guinea pigs to systemic endotoxin. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1635–R1645, 1999.—We reported recently that the complement (C) system may play a role in the febrile response of guinea pigs to intravenous lipopolysaccharide (LPS) administration because C depletion abolished the LPS-induced rise in core temperature (Tc). The present study was designed to investigate further the relation between C reduction [induced by cobra venom factor (CVF); 20, 50, 100, and 200 U/animal iv] and the fever of adult, conscious guinea pigs produced by LPS injected intravenously (2 µg/kg) or intraperitoneally (8, 16, 32 µg/kg) 18 h after CVF; control animals received pyrogen-free saline. Serum C levels were measured as total hemolytic C activity before and 18 h after CVF injection and expressed as CH100 units. In other experiments, serum C levels were determined at various intervals after the intravenous and intraperitoneal injections at different doses of LPS alone. LPS produced fevers generally of similar heights but of different onset latencies and durations, depending on the dose and route of administration. CVF caused dose-related reductions in serum C, from ~1,136 U to below detection. These reductions proportionately attenuated the fevers induced by intraperitoneal LPS, but not by intravenous LPS. Intravenous and intraperitoneal LPS per se caused reductions in serum C of 25 and 40%, respectively, indicating activation of the C cascade. These decreases were transient, however, occurring early during the febrile rise ~30 min after LPS injection. These data thus support the notion that the C system may be critically involved in the febrile response of guinea pigs to systemic, particularly intraperitoneal, LPS.

Anaphylatoxins; lipopolysaccharides; macrophages; interleukin-1; tumor necrosis factor; prostaglandin E2

In guinea pigs, a characteristically biphasic fever is induced within 10–15 min after the intravenous injection of lipopolysaccharide (LPS; the protein-free form of bacterial endotoxin) at a dose of 2 µg/kg, concomitantly with an increase in the levels of both circulating and brain PGE2 (a putative mediator of fever; Ref. 4). It is now generally thought that these effects are not mediated by LPS itself, but by secondarily host-derived cytokines or endogenous pyrogens, among which tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 are considered to be particularly important (12). These are thought to be produced mainly by mononuclear phagocytes that release them into the bloodstream for transport to and action in the brain, specifically the preoptic area (POA) of the anterior hypothalamus (4). A difficulty with this notion, however, is that the first of these cytokines to be released, TNF-α, is not detectable in blood exiting the liver (the major source of these cytokines in response to circulating LPS) until, at the earliest, 30 min after the intravenous injection of LPS (19, 25). Moreover, very recently, evidence has been adduced that TNF-α may not have a role in the initiation (first phase) of LPS fever in guinea pigs, but only in its maintenance (second phase) (41). This would suggest, therefore, that circulating cytokines may not be the direct stimuli that initiate the body temperature and preoptic PGE2 rises in response to intravenously injected LPS. But, on the other hand, threshold concentrations of relevant pyrogenic cytokines could be reached in the vicinity of their producing cells and activate appropriate local sensors, if they existed, well before these cytokines would be detectable in the general circulation. Indeed, the rapidity of the febrile response to intravenous LPS would imply a neural rather than a humoral communications pathway between peripheral endogenous pyrogens and the POA.

Because circulating LPS is cleared primarily by hepatic macrophages (Kupffer cells (Kc); Ref. 32), it is conceivable that vagal afferents originating in the liver may convey their released pyrogenic messages to the brain. In support, bilateral truncal subdiaphragmatic vagotomy (40, 43, 54) and, more particularly, selective hepatic vagotomy (45), block the febrile responses of guinea pigs and rats, respectively, to intravenous LPS. However, whereas the binding of LPS to its receptors in Kc occurs very rapidly, the subsequent half-maximal induction time of TNF-α requires a minimum of 20 min of contact with LPS in vitro (29); the half-maximal induction time of the other pyrogenic cytokines is still longer, i.e., longer in all cases than the latency of fever onset. We considered, therefore, whether the stimulus for Kc cytokine production might be provided not by LPS per se but by secondary mediators occurring in almost immediate reaction to the presence of LPS with blood. The intravenous administration of LPS triggers within 2 min the complement (C) cascade via both the classical and alternative pathways, resulting in the production in blood of the anaphylatoxic C fragments, C4a, C3a, and C5a, and of surface-bound and fluid-phase C3b and iC3b (reviewed in Ref. 52). Kc express the receptors for various C-derived fragments (23), and the production of cytokines and PGE2 by Kc and other phagocytes is initiated after their addition (1, 7, 22a, 39). Consequently, we hypothesized that the rapid onset of intravenous LPS-induced fever may be mediated via intravascular C activation by LPS and subsequent Kc stimulation by C anaphylatoxins. In support, we reported recently (44) that guinea pigs rendered hypocomplementemic or Kc deficient by prior treat-
ment with cobra venom factor (CVF) or gadolinium chloride, respectively, exhibited body core temperature (Tc) falls instead of rises and greatly attenuated elevations of preoptic PGE2 after intravenous LPS compared with untreated controls. Thus the onset of LPS fever may indeed critically depend on the activation of intravascular C and the subsequent stimulation of Kc by C fragments.

Although C was implicated previously in the fever caused by antigen-antibody complexes (34), to our knowledge ours was the first demonstration of its possible involvement in LPS-induced fever. There are no consistent reports in the literature, however, that human patients hereditarily deficient in one or another anaphylatoxic C do not fever normally during infections of various etiologies, albeit the severity of their effector responses is often attenuated (37). To preclude, therefore, that our finding might have represented only an unusual situation, the present study was designed to verify, under more detailed conditions, the putative relationship between C and the fever of guinea pigs produced by LPS. To this end, we injected graded doses of LPS intravenously or intraperitoneally and compared their effects on the febrile courses of animals with different degrees of CVF-induced complementation and, in other experiments, on the plasma C levels of C-sufficient animals at various intervals over the duration of their fevers. The results suggested a greater dependence on C when LPS was injected intraperitoneally than when it was administered intravenously.

METHODS

Animals

Male Hartley guinea pigs (301–350 g; Charles River Laboratories, Wilmington, MA) 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 (Ta) in the animal room was 23 ± 1°C. The Tc of the guinea pigs were conscious, were loosely restrained in their individual wire mesh cages at Tc 23 ± 1°C. The Ta of the guinea pigs were monitored constantly and recorded at 2-min intervals for the duration of the experiments on a Macintosh Plus IMb microcomputer through an analog-to-digital converter using precalibrated copper-constantan thermocouples inserted 5 cm into the colon. The data were displayed digitally on a monitor, printed on an ImageWriter printer, and stored on a diskette. A 90-min stabilization period to achieve thermal equilibrium preceded all the measurements. To obviate possible effects of circadian variations, the experiments were begun at the same time of day (0830).

Assay of Serum Total Hemolytic C Activity (CH100)

At fixed intervals, blood (200 µl) was withdrawn through the venous catheter, clotted in ice for 30 min, and centrifuged (3,000 rpm, 4°C, 10 min) to obtain the serum. The samples were then stored at −70°C until assayed. For an assay, 5 µl of serum were added to wells placed in agarose gel containing standardized sheep erythrocytes sensitized with hemolysin (kit 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 (radial immunodiffusion) were measured by imaging these zones with a charge-coupled device (CCD) camera, then scanning the images and calculating their relative optical densities using the National Institutes of Health (NIH) Image version 1.61 for analyzing electrophoretic gels. These values were converted to CH100 units (one unit being the amount of C that lyses 100% of the erythrocytes) by interpolation from calibration curves plotted using the manufacturer’s standard, diluted from neat to 1:32 (minimum sensitivity, 32 CH100 units). Experiments

The following experiments were conducted. Experiment 1. To determine, before all the other experiments, the conditions that would achieve graded, sustained reductions in serum C levels, guinea pigs received through their preinserted venous catheters either PFS (0.6 ml) or, initially, 20 U of CVF in 0.6 ml of PFS per animal; blood samples were collected for serum CH100 assays at 6, 12, 18, and 24 h and daily for the following 5 days thereafter. Because this experiment established that 18 h post-CVF was a suitable interval to achieve the desired effect (please see RESULTS), in the subsequent dose-response tests, 50, 100, and 200 U/animal were injected (the latter 2 doses in 2 boluses, namely, the first bolus at 50 U and the remaining dose in a second bolus delivered 2 h later to minimize the acute effects of complementation; Ref. 44), and blood was withdrawn at 18 h only.

Experiment 2. To determine the effect of different degrees of hypocomplementemia on the febrile response to LPS, guinea pigs were injected through their intravenous catheters with...
PFS or CVF at 20, 50, 100, or 200 U/animal, as in experiment 1. Eighteen hours later, they received, either through the same catheters or via sterile 23-gauge needles directly into the peritoneum, a bolus injection of 0.2 ml of PFS or, respectively, 2, 8, or 16 µg of LPS (in 0.2 ml of PFS)/kg body wt. Blood was withdrawn immediately before the LPS administration to assess the serum total hemolytic C activity. These intraperitoneal doses were chosen on the basis of preliminary dose-response studies designed to determine those over the range from 2 to 32 µg/kg body wt. Blood was withdrawn immediately before the LPS administration, we injected 0.3 ml of FITC-labeled phages preponderately in the liver, irrespective of its route of administration, we injected 0.3 ml of FITC-labeled Escherichia coli LPS (Sigma Chemical, St. Louis, MO; 3 µg/µl of PFS and at 0, 15, 30, 60, 120, and 360 min after intraperitoneal PFS or LPS and at 0, 15, 30, 60, 120, and 360 min after intraperitoneal PFS or LPS.

Experiment 3. To determine whether the small pyrogenic doses of LPS delivered in the preceding experiments do in fact, like larger doses (52), activate the C cascade, i.e., induce the consumption of C, guinea pigs were injected through their preinserted venous catheters or by needle into the peritoneum with, respectively, 2, 8, or 16 µg of PFS or, respectively, 20, 160, or 320 U of LPS (in 0.2 ml of PFS)/kg body wt. Blood for C activity measurements was sampled at 0, 2, 5, 10, 20, 30, 120, and 360 min after intravenous PFS or LPS and at 0, 15, 30, 60, 120, and 360 min after intraperitoneal PFS or LPS.

Experiment 4. Because in the present study the effect of 200 U of CVF on the febrile response to 2 µg iv of LPS/kg was different from that observed in our previous study (44) in which the guinea pigs were instrumented with, in addition to intravenous catheters, stereotaxically preimplanted intraportal microdialysis probes, an a posteriori experiment was conducted in which the responses of animals with and without such intracerebral devices were compared. Thus guinea pigs were prepared exactly as described under Surgery above, i.e., with an intravenous catheter alone or according to the procedure described previously (44), with, first, stereotaxically implanted guide cannulas in the left medial POA followed 4 days later by the insertion of the catheters into the jugular vein. CVF (200 U/animal) was delivered intravenously in the nonprobe group 7 days and in the probe group 10 days after the only or first surgery, respectively, and LPS (2 µg/kg) was injected intravenously 18 h later. No blood was collected in this series.

Experiment 5. To verify whether LPS activates macrophages preponderantly in the liver, irrespective of its route of administration, we injected 0.3 ml of FITC-labeled Escherichia coli LPS (Sigma Chemical, St. Louis, MO; 3 µg/µl of PFS/animal; this dose is in excess of pyrogenic doses in group 10 days after the only or first surgery, respectively, and LPS (2 µg/kg) was injected intravenously 18 h later. No blood was collected in this series.

Statistical Analyses
The results are reported here as means ± SE. The values of Tc are changes from basal values (Tc, initial), the Tc at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period) plotted at 6-min intervals. Fever indexes were derived from the area under the 6-h fever curve by imaging with a CCD camera, then scanning the images using NIH Image version 1.61. These values were converted to degree Celsius per hour by interpolation from a calibrated standard area that was 1°C high and 1-h long. Serum total hemolytic C activities were determined as absolute CH100 units and are expressed here as relative changes normalized to their initial values. Student’s paired t-test was used to compare pre (initial Tc, CH100, etc.) and post (maximal Tc, CH100, etc.) values within treatment groups. Differences in Tc or CH100 between treatment groups 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 was the within-subject factor (the different sampling periods). Each variable was considered to be independent. The strength of correlation between serum C reductions and fever indexes was calculated from the regression lines. Latencies of fever onset were determined as the intervals (in min) between the time of LPS injection and that of the first Tc rise that continued uninterruptedly >0.5°C. The 5% level of probability was accepted as statistically significant.

RESULTS

Experiment 1
The intravenous administration of CVF very rapidly caused a profound reduction in the guinea pigs’ serum CH100 activities from their initial 1,136 ± 67 U level (Fig. 1B). The extent and duration of this reduction were both dose dependent, that is, CH100 decreased to...
its lowest level commensurate with the given CVF dose within 6 h, then progressively recovered over the following 6 days, the rate of recovery being slower the higher the dose. This is illustrated for the lowest dose of CVF tested, 20 U/animal, in Fig. 1A. To minimize potential circadian effects on the variables being measured, we traditionally begin our experiments at 0830, administering the first treatment 90 min later, i.e., at ~1000 (please see METHODS). On the basis of the above findings, we selected 18 h post-CVF as the time point to be coincident with the 1000 treatment in the subsequent studies. Hence, CVF was injected at 1600 on the previous day. The effects of various doses of CVF on serum CH100 activities extant 18 h after drug administration are illustrated in Fig. 1B. The variations are due to differential sensitivities inherent among the animals and divergences among the lots of CVF and CH100 assay plates received.

Experiment 2

LPS intravenously at 2 µg/kg induced characteristically biphasic, ~1.3°C, fevers with onset latencies of ~11 min (Fig. 2B, PFS). The first febrile peak occurred ~48 min after LPS administration and the second 84 min after the first. The return to Tc values was gradual but essentially completed by 270 min after LPS injection. The Tc of the guinea pigs given PFS intravenously did not vary significantly over the same 6-h duration (Fig. 2A, PFS). CVF given intravenously 18 h before similar had no apparent effect on the Tc of the guinea pigs that received PFS subsequently (Fig. 2A, CVFs). Likewise, pretreatment with CVF did not demonstrate alter the febrile response to LPS of these animals (Fig. 2B, CVFs), albeit it appeared that at one CVF dose, 50 U/guinea pig (Fig. 2B, CVF 50), both the first and second febrile rises were attenuated and the postfebrile recovery stabilized at a lower level than that of the PFS group. In view of this disparity, this series was replicated; hence, the larger n value. However, the onset latency, time to first peak, and febrile course proved not significantly different in this larger group compared with the other LPS-treated groups. Higher LPS doses were not tested in this phase of the experiment, because 2 µg/kg is already a nearly maximally effective pyrogenic intravenous dose in guinea pigs (see Ref. 3).

Although the fever heights produced by intraperitoneal LPS were similar for all the doses we tested (2, 4, 8, 16, and 32 µg/kg), the patterns and durations of the fevers were different, such that the fever indexes increased progressively at 2 and 4, were maximal at 8, then decreased again at 16 and 32 µg/kg (not illustrated). The onset latencies, on the other hand, steadily decreased as the LPS doses were increased. These data are summarized in Table 1. Although the onset latency of intraperitoneal LPS at 8 µg/kg was significantly longer than that of intravenous LPS at 2 µg/kg, its other characteristics were quite similar (Figs. 2B and 3B, PFS, respectively). We therefore chose this dose as the standard for our subsequent intraperitoneal LPS studies.

Figure 3 depicts the effects of intraperitoneal PFS (Fig. 3A) and LPS (Fig. 3B) on the Tc of the animals pretreated intravenously with PFS or the four doses of CVF employed 18 h before their injections. As before, the CVF pretreatment had no demonstrable effect on
the Tc of the animals that received PFS. On the other hand, it dose dependently reduced (20, 50, and 100 U) or completely prevented (200 U) the LPS fever. Indeed, both the change in maximal Tc (not illustrated) and the fever indexes (Fig. 3C) of the CVF-pretreated conscious guinea pigs after intraperitoneal administration of LPS (8 µg/kg).

To characterize further the susceptibility of intraperitoneally injected LPS fever to decomplementation, guinea pigs were injected intraperitoneally with 16 µg·LPS−1·kg body wt−1·h after CVF at 50, 100, and 200 U. The fevers evoked were quite similar to those produced by 8 µg/kg (Fig. 3B, PFS), except that the onset latency after the larger dose was shorter than after the lower (Fig. 4, PFS and Table 1). Whereas, as described above, CVF pretreatment from 20 U upward dose dependently both delayed the onset and reduced the height of the febrile response to 8 µg of LPS/kg ip, it required 100 U of CVF to begin to effect the same result (Fig. 4, CVFs), i.e., the higher the endotoxic stimulus, the more resistant the fever to C reduction.

Experiment 3

The fevers produced by intravenous LPS at 2, 8, and 16 µg/kg were essentially similar; the response to 8 µg/kg is illustrated in Fig. 5A. This dose caused the Tc to rise with the quickest onset and the highest first maximum. It also produced a significant, ~40%, decrease in serum CH100 activity 30 min after its administration (Fig. 5B). This dose, however, had no effect on C levels at any other sampled time over the febrile course. Two and sixteen micrograms of LPS per kilogram body weight, on the other hand, did not induce statistically significant changes in CH100 at any time (not illustrated). The intravenous injection of PFS caused no change in Tc and CH100 over the same time course.

LPS intraperitoneally at 8, 16, and 32 µg/kg induced fevers with progressively shorter latencies and different patterns, but essentially similar maxima, as already described (Table 1); the response to 8 µg/kg is depicted in Fig. 5A. No changes in serum CH100 activities were noted in response to these treatments, except a 25% reduction at 30 min after 8 µg (Fig. 5B). The similarities in the profiles of CH100 activities after 8 µg of LPS intravenously and intraperitoneally are noteworthy. PFS intraperitoneally had no effect on either of these measured variables.
Experiment 4

In confirmation of our previous identical experiments (44), the febrile responses of guinea pigs pretreated with 200 U of CVF intravenously 18 h before receiving 2 µg of LPS/kg iv were abolished and converted into 0.9°C hypothermic responses if the animals had been prepared 10 days before CVF administration with unilaterally implanted intrapreoptic microdialysis probes, but not altered if their brains were intact (Fig. 6). Thus the integrity of the POA would seem to be a determinant of the direction of the thermal response to intravenous LPS in otherwise similarly hypocomplementemic guinea pigs. The febrile responses to LPS of C-sufficient guinea pigs were not influenced by the preimplantation of microdialysis probes unilaterally into the POA compared with intact animals (not illustrated; see Ref. 44).

Experiment 5

FITC-labeled LPS was detectable as patches of granular fluorescence 15 min after its intravenous injection within, presumptively, aggregated Kc in the liver sinusoids (Fig. 7B). "Presumptively" because, due to a difficulty inherent in the confocal microscope itself, that is, despite its high resolution, fluorescence emitted above or below the focal plane results in blurs and halos, impeding discerning whether the fluorescence was in or on the Kc; this difficulty is compounded when the labeled cells are aggregated. At 60 min, fluorescent patches also appeared in the hepatocytes (Fig. 7C). In stark contrast, no FITC labeling appeared at 15 and 60 min in any part of the guinea pigs' livers after intraperitoneal FITC-LPS (Fig. 7, E and F). Only normal autofluorescence was observed after the intravenous or intraperitoneal administration of fluorescein sodium salt at a dose equivalent to its amount in the FITC-LPS conjugate (Fig. 7, A and D).

DISCUSSION

The reduction in C in this study was accomplished, as in our previous study (44), by use of CVF, which activates the alternative pathway of the C cascade (10). It forms a complex with factor B, CVF Bb, which is functionally analogous to C3b Bb, the natural C3 convertase that cleaves catalytically the α-chain of C3. The difference between the two compounds is that CVF Bb is highly resistant to the normal control mechanisms that limit the activity of C3b Bb, so that fluid-phase C activation continues unabated, drastically depleting C. Consequently, absent the substrates from which they are produced, all the subsequent C components are also depleted; hence, hypocomplementemia results. Since the present data showed, in confirmation of our earlier observations (44), that CVF-induced hypocomplementemia, as indicated by a decreased serum CH100 activity, impaired the febrile response of conscious guinea pigs to systemic LPS, some or all of the fragments from C3 to C9 must be important for fever production, at least in guinea pigs. Unexpectedly, however, this dependence on the C system was contingent, in turn, on the route of LPS administration; namely, the febrile response was highly C dependent when LPS was delivered intraperitoneally, but apparently not so when it was injected intravenously. The reason for this dichotomy is not immediately obvious; an attempt to explicate it will be made later in this section.

It is well established that LPS very rapidly activates the C cascade (52). Lipid A initiates the classical pathway, and the polysaccharide A initiates the alternative pathway, and the polysaccharide A initiates the alternative pathway.
alternative pathway. Among the C components consequently generated, C3a, C3b, iC3b, C5a, C5a (desArg) and the membrane attack complex (C5b-9, in sublytic concentrations) are of particular interest in our context. Among their multiple inflammatory activities, they independently induce the production of TNF-α, IL-1β, IL-6, and PGE2 by phagocytic and other cell types (1, 7, 22a, 39, 47). They also amplify the secretions of TNF-α and IL-1 induced by LPS (7). It may be logically assumed, therefore, that to the extent that the production of these pyrogenic mediators may be impaired in C-insufficient animals, their febrile responses will be correspondingly attenuated. Indeed, the involvement of C in LPS-induced host defense responses is well documented, and the consequent C activation is associated with the consumption of C components so that a reduction in their blood concentrations is observed in many inflammatory diseases (37). Therapeutic inhibition of C similarly has been shown to arrest the process of certain diseases (26). The reductions in serum CH100 observed in the present study after both intravenous and intraperitoneal LPS (Fig. 5) thus lend support to an important role for C in fever production, albeit a limitation of measurements of C levels in serum for assessing its activation is that only very marked reductions can be reliably quantified by this technique because C factors are synthesized and degraded at a very high rate. Consequently, increased utilization of C caused by pyrogenic doses of LPS is difficult to evaluate precisely within this background of rapid turnover.

Only few studies have been conducted as yet, however, that examined the possible role of C specifically in fever production. In one study of human subjects injected intravenously with low doses of E. coli LPS, no changes in anaphylatoxin levels were detected in the plasma, although Tc and plasma TNF-α and IL-6 levels rose after 30–45 min (50). But in another study in humans, the expression of C3b and iC3b receptors (CR1 and CR3) on neutrophils was significantly augmented (36). Upregulation of CR3 was also seen on rabbit neutrophils within 5 min after exposure to LPS, culminating within a further 25 min (30). Mickenberg et al. (34) reported that the total hemolytic C activity and C3 titers of rabbits fell within 5 min after the intravenous administration of low-dose, soluble antigen-antibody 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. These data conform with our own observations using LPS. To the degree that fever is a manifestation of the hosts’ reactions to infectious stimuli, its reduction under these conditions is in line with the loss of other host...
defense functions that are normally coactivated with fever. These include the inability of C-deficient animals to enhance phagocytosis, promote chemotaxis, and release various inflammatory mediators in response to infection (37), albeit congenitally C-deficient, clinically infected patients can present with fever (37). However, the pathogenesis of clinical infections is more complicated than represented by the present study, generally involving, in addition to LPS, multiple other factors not incorporating C mediation.

Because it has been demonstrated in vitro that C components trigger the production of cytokines and PGE₂ by macrophages, we had hypothesized that the rapid onset of intravenous LPS-induced fever may be mediated via the intravascular activation of C by LPS and the subsequent stimulation of Kc by C-derived fragments to release these mediators. Kc were favored over other macrophages in this context because the hepatic macrophages are quantitatively the most important (Kc constitute 80% of all resident mononuclear phagocytes in the body) and the liver is the principal source of cytokines liberated into the bloodstream. Other data pointing to Kc included, in conformity with the neural hypothesis of fever induction (5, 31), recent findings that hepatic vagal branch transection inhibits the febrile responses of rats to intravenous LPS (40, 44, 54), that putative IL-1 receptors exist on hepatic afferent fibers (21), and that their cell bodies express c-fos in response to intravenous LPS (18). It was surprising, therefore, that the fevers induced by intravenous LPS were evidently C independent (Fig. 2B), whereas those produced by intraperitoneal LPS were dose dependently sensitive to C reduction (Fig. 3, B and C). There is evidence, however, that the activation of macrophages by LPS for synthetic responses may proceed by various pathways. Thus CD14 is the predominant LPS receptor on macrophages. Its activation requires that LPS complexes to LPS-binding protein (LBP), which is present in normal plasma but absent in blood-free peritoneal fluid (13, 48). Indeed, only minimal amounts of LPS-induced cytokines and PGE₂ are released in vitro from macrophages in general in the absence of LBP. If LPS-CD14 interactions are not favored in the peritoneal fluid because of the absence of LBP and yet fever develops after intraperitoneal LPS, it may be surmised that a different LPS signaling system may activate peritoneal macrophages, one that, according to the present data, may be critically dependent on C. Such a system may be provided by complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which have been shown also to function as LPS transmembrane receptors that activate macrophages (24). They recognize iC3b-opsonized particles, and binding sites for LPS and for iC3b coexist in the same molecule (56), thus mediating the attachment of iC3b-coated LPS to macrophages and inducing IL-1β and TNF-α synthesis (8). Hence, we speculate that C fragments, LPS, and iC3b-opsonized LPS, in tandem, may coactivate peritoneal macrophages through these signaling pathways. A decrease in C fragments for opsonization of LPS would be expected, therefore, to concentration dependently impede the ability of these macrophages to respond to LPS and to C-derived components. It is also conceivable, alternatively, that the activation of CR3 and CR4 contributes under these conditions to the migration and adhesion of peritoneal macrophages to counter-receptors on other cognate cells, perhaps in perivagal paraganglia (20); cell-to-cell contact could be a requirement for the subsequent (1 h) induction of IL-1β by such cells or for its binding to them (21). It may be pertinent in this context that peripheral nerves express C receptors (51). By contrast, in the bloodstream, C insufficiency may be less consequential than in the peritoneal cavity because of the presence of LBP in plasma, allowing LPS-charged CD14 to generate fever-promoting signals. If substantiated, this speculative accounting for the differential activation of intravascular and tissue macrophages by LPS would provide functional verification of in vitro findings that, as yet, have no defined in vivo corollaries.

It may thus be inferred from the preceding that TNF-α and IL-1β could be released by peritoneal macrophages in consequence of the activation of the C cascade by LPS. However, their production by these cells requires their transcription and translation, and the time course of their synthesis in vitro is evidently not any more rapid in response to C fragments than to LPS stimulation, namely, 1–2 h (7, 8, 22a, 29). In vivo, LPS administered intraperitoneally to rats induced the expression of IL-1β protein in various immune cells surrounding abdominal vagal afferents in 45–60 min (20), i.e., quicker than in vitro. Although this interval was roughly commensurate with the onset latencies of the fevers thus produced in rats, it was longer than those produced in our guinea pigs by intraperitoneal LPS at doses of 8 µg/kg or greater (Table 1). This delay would suggest, therefore, at least insofar as guinea pigs are concerned, that, to the extent that C-derived fragments could mediate the eventual, enhanced release of pyrogenic cytokines after LPS (7, 8), their role may not be in the initiation of the febrile response to intraperitoneal LPS but rather in its subsequent maintenance. On the other hand, because the onset of these fevers was delayed and their initial rises were attenuated in proportion to the degree of hypocomplementation, C would appear also to contribute to the initiation of the febrile response to intraperitoneal LPS. In view of the short time frame in this study between LPS injection and fever onset, we would suggest that the febrigenic triggering mediator thus arising in the peritoneum of guinea pigs may be constitutively expressed rather than induced de novo. Mast cells are other peritoneal cell types also responsive to C fragments. Although they reportedly contain preformed TNF-α (22), which could, therefore, be quickly released for binding to local sensory vagal terminals, it was recently shown (41) that TNF-α probably does not contribute to the initiation of the febrile response of guinea pigs to LPS; rather, its role also appears to be associated with the subsequent maintenance of fever. This would conform with our earlier suggestion.
LPS fever and complement

PGE$_2$ is another product of macrophages that could rapidly provide the initial signal for fever onset. Thus C3a and C5a stimulate PGE$_2$ production by, e.g., hepatic macrophages, within 2 min (39). CVF pretreatment prevents the rise both in plasma and in intra-POA PGE$_2$ induced by intravenous LPS (14, 44), and EP$_3$ receptors have recently been specifically implicated in the febrile response (49); the latter exist in all the sites where the transduction of peripheral pyrogenic messages has been proposed to occur (5, 46). There is a caveat, however: the proposed involvement of PGE$_2$ would be possible only if the production of PGE$_2$ by C were cyclooxygenase (COX)-2 mediated, because we (28) and others (reviewed in Ref. 33) have demonstrated that LPS-, IL-1$\beta$-, and TNF-$\alpha$-induced fevers are strictly dependent on the mediation by this isozyme of PGE$_2$ synthesis. Because COX-1 null mutant mice develop normal fevers in response to LPS (28) and COX-2 is not constitutively expressed in unstimulated macrophages, it follows that PGE$_2$ is probably not the triggering signal to local neural afferents. Hence, another factor, quickly released by as yet unspecified C-responsive cells in the vicinity, could provide this signal, but its identity remains elusive.

The distinct uptake of LPS by Kc depending on its route of administration was somewhat unexpected because numerous previous studies had shown that LPS injected intraperitoneally was recovered in the bloodstream and cleared primarily by the liver. However, this evidence was based on studies in which relatively large doses of LPS were injected (11, 42). Nevertheless, it may be assumed from the present results that the population of phagocytes that accomplishes LPS uptake depends on the dose and route of its administration. Thus, after jugular vein injection, as in this study, LPS appears suddenly at its full dose in the bloodstream. Although pulmonary intravascular macrophages constitute the first filter encountered, the rate of LPS clearance and detoxification by these cells is very slow in rodents (53), so that it overflows into the general circulation. Consequently, neutrophils, monocytes, and other phagocytes within the vasculature, namely, resident sinusoidal hepatic and splenic macrophages, effect the intravascular clearance of LPS; of these, the Kc are believed to assume the greater role (40%) (32, 42). Small percentages of LPS are also taken up by the kidneys, adrenal glands, lungs, and skeletal muscle (17). Kc internalize LPS by absorptive pinocytosis and cleave the sugar side chains of the core oligosaccharide to a uniform length such that the molecule can be passed to hepatocytes for final disposition (14). Our present finding that FITC-LPS is detectable in the liver sinusoids within 15 min and in hepatocytes within 60 min after its intravenous injection corroborates those earlier studies. Despite the effectiveness of this filter, however, because only about one-third of the cardiac output is distributed to the splanchnic organs, intravenously injected LPS recirculates to the liver only one-third of the time and, consequently, in larger doses partially evades first-pass hepatic clearance. Extrahepatic macrophages then also contribute to removing the spillover. This may account for the observations that subdiaphragmatic vagotomy is effective in inhibiting fever only when the intravenous LPS dose is small (40). Intraperitoneal LPS, on the other hand, is thought to be removed principally by way of the lymphatic channels that rest under the diaphragmatic mesothelium. Inspiratory and expiratory lymphatic movements open and close the specialized stomata that provide access from the peritoneal cavity to the lymphatic lacunae. These lead to larger intrathoracic lymphatic channels, which, in turn, enter the thoracic duct and drain into the subclavian vein (11). Therefore, in contrast to intravenously injected LPS, intraperitoneally injected LPS appears progressively in the blood. Concomitantly, intraperitoneal LPS is taken up by resident peritoneal macrophages and by macrophages in lymph nodes by receptor-mediated endocytosis and degraded by deacylation of the fatty acids of the lipid component (15, 29); obviously, this portion of the dose injected does not pass into the circulation. Indeed, if the intraperitoneal LPS dose is small, no LPS at all may appear in the circulation (6, 27). This was evidently the case in the present experiments, because no FITC-LPS was detectable in the liver at 15 and 60 min after its intraperitoneal injection.

It is not clear why the same amount of decomplexed attenuated the fevers of the guinea pigs with intraperitoneally implanted microdialysis probes, but did not affect the febrile responses of the brain-intact animals (Fig. 6 and Ref. 43). No plasma C reaches the brain when the blood-brain barrier is intact. Hence, its reduction in plasma should be without effect on the brain. But both C mRNAs and their receptors are significantly upregulated on reactive astrocytes and microglia in response to local injury (38), such as would result from the insertion of a microdialysis probe, as in this study. Although other evidence suggests that the thus breached blood-brain barrier rescales in several hours (2), it is possible that some new capillaries in the region may have a reduced blood-brain barrier and allow plasma proteins to pass into the brain parenchyma and cerebrospinal fluid (35). In that case, CVF might have gained entry and prevented the expression of C by reactive glial cells. It is also possible that the CVF pretreatment per se provoked vascular leakage into the gliotic area surrounding the probe, thus allowing its own passage into the POA and depleting local reactive microglia and other macrophages of C and perhaps, consequently, also downregulating their expression of C receptors. Whatever the mechanism, however, these results would suggest that brain C may also have a role in LPS fever genesis.
onset, we speculate that these elicited cytokines are probably not the initial febrigenic trigger. Whether the same factor occurs in the peritoneal fluid and in the bloodstream and whether it provokes the same or different signals also remains to be clarified. But regardless of the particular factor of the C cascade responsible or of its mechanism of action, these data show that C deficiency is associated with lower fevers, indicating a contribution of the C system to the development of intraperitoneal LPS-induced fever.

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