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Complement Component C5a Is Integral to the Febrile Response of Mice to Lipopolysaccharide

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Key Words

C3 null mutants · C5 null mutants · Complement receptors · Complement 5a receptor antagonist · Cobra venom factor · Pyrogenic cytokines

Abstract

Objectives: The complement system is critical to the febrile response of mice to intraperitoneally administered lipopolysaccharide (LPS). We previously identified C3 and C5 as two components potentially involved in this response. This study was designed to examine whether the complement system is also pivotal in the response of mice to intravenously or intracerebroventricularly injected LPS, to distinguish between C3 and C5 and their cognate derivatives as the essential mediator(s), and to determine whether the failure of complement-deficient mice to develop a fever could be due to their possible inability to secrete pyrogenic cytokines. ***Methods:*** Wild-type (WT; C57BL/6J) mice, hypocomplemented or not by intravenously injected cobra venom factor (10 U/mouse), and C3-, CR3- and C5-sufficient and -deficient mice were intravenously challenged with LPS (0.25 μg/mouse); WT and C3–/– mice pretreated with a C5a receptor antagonist (C5aRa) were similarly challenged. In addition, the serum levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 were compared in LPS-treated C5+/+ and C5–/– mice. ***Results:*** LPS induced a 1°C rise in core temperature in all the mice, except C5–/– mice and those pretreated with C5aRa. C5+/+ and C5–/– mice challenged intracerebroventricularly with LPS exhibited identical febrile responses. LPS induced similar increases in the serum levels of IL-1β, TNFα and IL-6 in C5+/+ and C5–/– mice. ***Conclusions:*** C5a is crucial for the development of febrile responses to LPS in mice; its site of action is peripheral, not central. The possibility that an inability to produce cytokines could account for the failure of C5–/– mice to develop a fever is not supported.

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Introduction

The administration of bacterial endotoxic lipopolysaccharide (LPS) induces a febrile response, the onset latency, magnitude, duration and pattern of which are dependent on the given dose, route of administration and time of day, and mediated by various pyrogenic cytokines, mainly tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and ultimately, prostaglandin (PG)E_2 [reviewed in ref. 1]. The presence of LPS in blood or tissue almost immediately activates the complement cascade, resulting

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standards established by the US Animal Welfare Act and by the documents entitled 'Guiding Principles for Research Involving Animals and Human Beings' [24].

Drugs

CVF *(Naja naja kaouthia)* was purchased from Calbiochem-Novabiochem (San Diego, Calif., USA). LPS was *Escherichia coli*, serotype 0111:B4 (lot No. 36F4019, prepared by trichloroacetic acid extraction; protein content 0.5%; Sigma-Aldrich, St. Louis, Mo., USA). PGE_2 (lot No. 77H1294; Sigma) was dissolved in pyrogen-free saline (PFS; 0.9% NaCl, USP; Abbott Laboratories, Chicago, Ill., USA) at 250 μg/ml. The vehicle for all the solutions was PFS. Murine C5 receptor antagonist (C5aRa) was synthesized by one of the authors (J.D.L.) [25]; the vehicle for this agent was sterile phosphate-buffered saline (PBS).

Injections

Intravenous Injection. On the experimental day, the previously trained mice were connected to the relevant measuring devices, placed in their confiners and allowed to stabilize for at least 3 h until their T_cs varied by not more than ± 0.1 °C over 5 consecutive 2-min periods. Treatment began after thermal stabilization was achieved. With the mice in their confiners and their tails extending outside the confiners, 0.1 ml of the desired drug or its vehicle was injected into a tail vein with a sterile 30-gauge × 1/2 needle and tuberculin syringe.

Intraperitoneal Injection. The mice were similarly stabilized as for the intravenous injection. Then they were removed from their confiners, gently held by hand, and 0.2 ml of the desired drug or its vehicle was injected into the peritoneum using a sterile 25-gauge × 5/8 needle and tuberculin syringe. The mice were then returned to their confiners.

Intracerebroventricular Injection. The intracerebroventricular injection procedure was modified from the methods of Haley and McCormick [26] and Kondo and Togari [27]. Briefly, the mice were lightly anesthetized with Metofane® (methoxyflurane, lot 012435; Schering-Plough, Union, N.J., USA), an ultrashort-acting inhaled anesthetic. Under aseptic technique, a 1-cm incision was made in the middle of the skin of the head, and the skull was exposed. A hole was quickly made with a 25-gauge needle above the right lateral cerebral ventricle localized by visual determination on the basis of its AP and L distances from the bregma (–1 and 1.5 mm, respectively) [28]. A sterile, stainless steel, 30-gauge cannula connected to a 10-μl Hamilton syringe via sterile PE-20 tubing was then inserted through the just opened hole; the tubing was fitted over the cannula so that precisely 2 mm of the cannula tip were exposed, allowing the cannula to pass through the hole into the skull (V: 1.5 + 0.5 mm for the skull), then to be stopped by the overfitted tubing. Five microliters of the pyrogens or their vehicles were injected over a period of 2 min while the animals were still under anesthesia; 2 min more were allowed to elapse before the injection cannula was removed, to avoid reflux. The hole was then sealed with bone wax and the skin was closed with tissue adhesive. The animals regained consciousness within 10 min. They were then immediately connected to their measuring devices, placed in their individual confiners, and the temperature recordings initiated; an interval of 30 min usually elapsed between the injection and the first measurement. No preliminary stabilization period preceded these recordings, since the handling associated with the injections causes immediate T_c rises that vitiate the value of basal T_cs. At the end of the experiments, the mice were euthanized with an overdose of Metofane, and their brains were removed and fixed overnight in 10% Formalin®. The injection cannula tracks were visualized the next day under a dissection microscope for verification of the injection locations. Only the data from mice with confirmed placement of the microinjection cannula into a lateral ventricle were included in this report. The authors have used this technique successfully in the past [14]; no untoward consequences are evident when it is skillfully performed.

Depletion of Complement

To achieve a maximum reduction of serum complement, based on our previous experiments in guinea pigs [13] and mice [14], 10 U of CVF/animal were injected into the mice's tail veins. To minimize possible acute effects of hypocomplementation, these doses were administered in two boluses. The first bolus of half the dose was given at 14.30 h, 21 h before the actual experiments; the second half of the dose was injected 2 h later. This schedule also served to minimize temporal deviations from our usual experimental protocol, i.e. beginning the experiments at 08.30 h of the following day.

Assay of Complement Activity

Due to the lack of readily available specific murine C3a and C5a protein assays, complement activity was evaluated as total hemolytic activity. At the conclusion of the experimental periods, the mice were deeply anesthetized with Metofane, and blood was collected from their orbital sinuses, clotted on ice for 30 min and centrifuged (Beckman Microfuge™ 12; 3,000 rpm, 4 °C, 10 min). The resulting serum samples were 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 No. RC003; The Binding Site, San Diego, Calif., USA). The plates were first incubated for 18 h at 4 °C, then for 1 h at 37 °C. They were photographed using a charge-coupled device camera, and the images were stored and printed using the NIH Image version 1.61 for analyzing electrophoretic gels. Since this kit is not as sensitive to mice serum complement as to guinea pig serum complement, it was not possible to assess the serum complement quantitatively. Therefore, only qualitative, visual determinations of the presence or absence of the complement were made (fig. 1).

Temperature Recording

The animals, fully conscious in their confiners, were placed under a plastic hood (free convection through open ports) to prevent undue disturbances from noise and fluctuations in T_a 23 ± 1 °C, their habitual housing T_a. It was chosen in preference to the mice's thermoneutral zone (26–34 °C) to obviate the hyperthermia that acute exposure to this environment induces in rodents not previously acclimatized to it [29]; this T_a was also previously used by the authors [30] and others [31]. The T_cs of the mice were monitored constantly and recorded at 2-min intervals for the duration of the experiments on a Macintosh Plus 1-Mb computer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 2 cm into the colon and taped to the tail. The data were displayed on a video monitor, printed digitally on an Imagewriter® printer and stored on a disk for subsequent statistical analysis. Control measurements were begun when the animals' T_cs had become stabilized, as described above. The treatment pertinent to a given experiment was then administered, and the measurements continued for the following 4 h according to the same routine as before treatment.

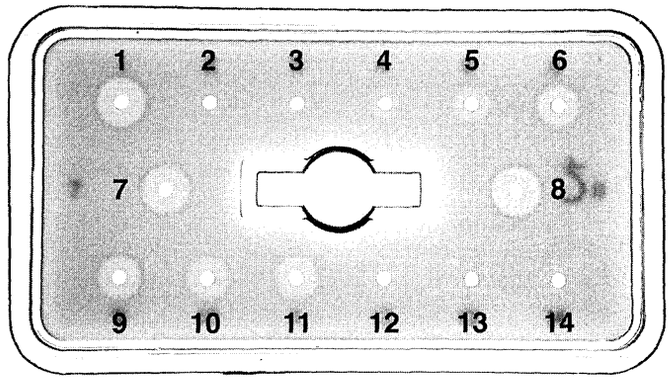

Fig. 1. The total hemolytic activity of mice serum complement. Well 1 represents the quality control. Wells 2–8 are dilutions of the standard (0, 34, 69, 138, 276, 551, 1102 CH_{100} U/ml, respectively), using the manufacturer's standard. Wells 9–11 illustrate positive reactions (complement is present) in serum from C57BL/6J (WT) mice. Wells 12–14 show no reaction (complement is absent) in serum from CVF-pretreated WT mice.

Serum Cytokine Measurements

Blood was collected by orbital bleeding under light Metofane anesthesia, and serum was prepared. IL-1β, TNFα and IL-6 levels were measured using ELISAs specific for murine cytokines from Pierce Endogen (Rockford, Ill., USA; No. EMIL-1B, No. EMTNFA and No. EM2IL-6) and carried out according to the manufacturer's instructions.

Experiments

Experiment 1: WT Mice Intravenously Injected with CVF/LPS. To establish whether the complement system may be involved in the febrile response of mice to intravenously injected LPS as it is in the response to intraperitoneally injected LPS [14], but not to intravenously injected LPS in guinea pigs [13], we administered CVF intravenously to WT mice; the control solution was PFS. Twenty-one hours later, the animals were injected intravenously with LPS [0.25 μg/mouse (~10 μg/kg) in 0.1 ml of PFS] or its vehicle (PFS, 0.1 ml/mouse). The present dose of LPS is in conformity with that in our [14] and others' [31, 32] previous studies. T_c was monitored continuously for 3 h before and 4 h following this injection.

Experiment 2: C3–/– Mice Intravenously Injected with LPS. To determine whether C3 may be involved in the fever produced by intravenously injected LPS as it is in that to intraperitoneally injected LPS [14], we administered LPS or PFS intravenously to C3–/– mice and measured their T_c responses before and after these treatments, as in experiment 1.

Experiment 3: CR3–/– Mice Injected Intravenously or Intraperitoneally with LPS. To determine whether the C3 derivatives C3b and iC3b may be involved in the fever induced by LPS, we measured the T_c of complement receptor-type-3-sufficient (CR3+/+) and -insufficient (CR3–/–) mice for 3 h before and 4 h following their challenge with LPS injected intravenously (0.25 μg/mouse) or intraperitoneally [1 μg/mouse (~40 μg/kg)]; the control solution was PFS (0.1 ml/mouse, i.v. or 0.2 ml/mouse, i.p.).

Experiment 4: C5–/– Mice Intravenously Injected with LPS. Following the same protocol as in experiment 2, we examined whether C5 may be pivotal to the febrile response to intravenously injected LPS by challenging C5–/– mice with intravenously injected LPS or PFS.

Experiment 5: WT or C3–/– Mice Injected Intravenously or Intraperitoneally with C5aRa and LPS. To determine whether the C5 derivative C5a may be the component mediating the febrile response to LPS, we intravenously injected C5aRa (1 mg/kg in 0.1 ml of PBS) or its vehicle (PBS, 0.1 ml/mouse) into WT or C3–/– mice immediately before the intravenous (0.25 μg/mouse) or intraperitoneal (1 μg/mouse) administration of LPS or PFS, and monitored the animals' T_cs before and after these treatments, as before.

Experiment 6: C5–/– Mice Intracerebroventricularly Injected with PGE_2 or LPS. To verify the integrity of the central mechanisms of the febrile response of C5-gene-ablated mice and determine whether brain C5 may be involved in the febrile response to LPS, PGE_2 (1.25 μg/mouse in 5 μl of PFS), LPS (50 ng/mouse in 5 μl of PFS) or PFS (5 μl/mouse) was microinjected intracerebroventricularly into C5+/+ and C5–/– mice under light Metofane anesthesia, as described earlier. The T_c recordings began 30 min after the injection and continued for 6 h.

Experiment 7. To determine the effect of C5 on the production of cytokines, PFS or LPS (0.25 μg/mouse) was injected intravenously into C5+/+ and C5–/– mice. Ninety minutes after the injections, blood was collected by orbital bleeding under light Metofane anesthesia. Serum was prepared and stored for assays of IL-1β, TNFα and IL-6, using murine ELISAs. T_cs of these mice were continuously recorded until the collection of blood samples.

Statistical Analyses

The results are presented as means ± SE. The values of T_c are reported as the changes from basal values – T_{ci} (initial), the T_c at 2-min intervals averaged over the last 10 min of the preceding 3-hour stabilization period. Student's unpaired t test was used to compare the maximal response to LPS and the response to PFS at the same time. Student's paired t test was used to compare pre (basal)- and post (the maximal LPS)-treatment data within a treatment. Differences between treatments were evaluated by a two-factor repeated-measures ANOVA (Microsoft Excel Analysis ToolPak), where factor 1 was the between-group 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

In all the mice, the intravenous injection procedure and the associated handling per se rapidly induced a transient, approximately 0.4°C rise in T_c, despite the prior training of these animals. This rise abated, however, in about 30 min, and then T_c gradually returned toward its initial level over the following 30 min in the PFS-treated group (fig. 2a). It then slowly decreased further by about 1°C over the following 3 h. LPS administration, on the

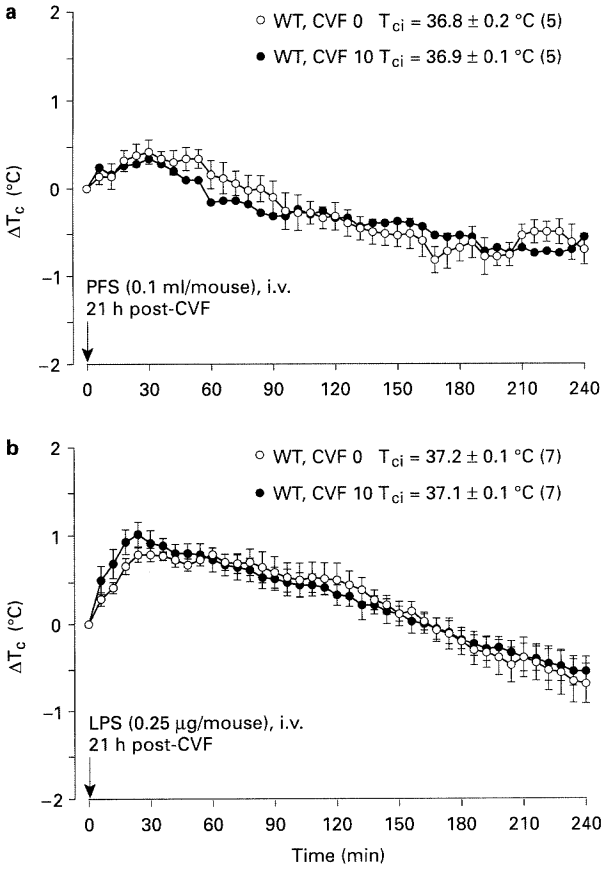

Fig. 2. Effects of intravenously injected PFS (0.1 ml; **a**) and *E. coli* LPS (0.25 µg/mouse in 0.2 ml PFS; **b**) on the T_c of C57BL/6J (WT) mice pretreated with PFS (CVF 0) or 10 U of CVF/mouse (CVF 10). The T_cs are expressed as differences (ΔT_c) relative to their initial levels (T_{ci}: average of the T_c over the last 10 min before the injection of PFS or LPS). CVF was given intravenously in two equally divided doses 21 and 19 h before these measurements; PFS or LPS was given at time 0. The values are means ± SE; n = number of animals.

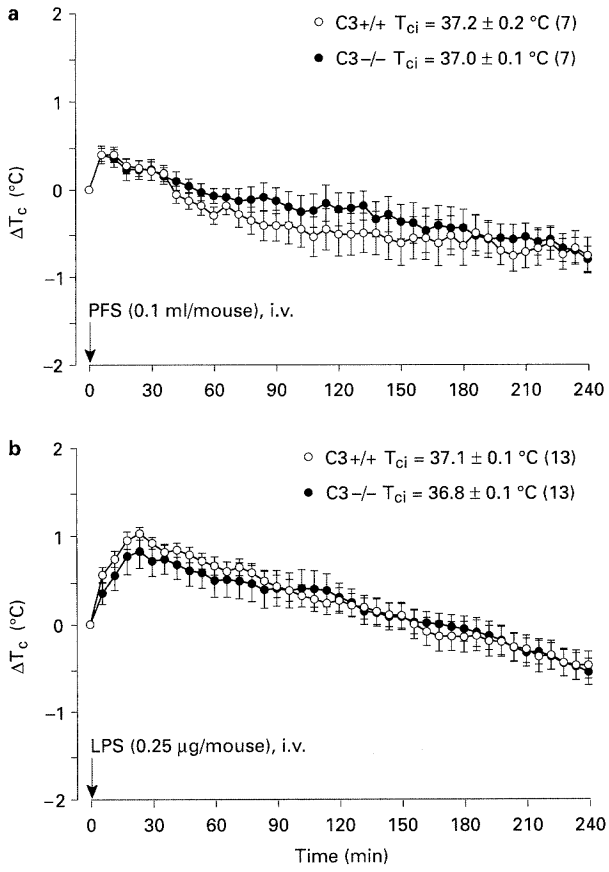

Fig. 3. Effects of intravenously injected PFS (0.1 ml; **a**) and *E. coli* LPS (0.25 µg/mouse in 0.2 ml of PFS; **b**) on the T_c of C3+/+ and C3–/– mice. Conditions and notations are as described in figure 2.

other hand, caused a further rise in T_c on top of the initial handling-induced rise. It peaked 1 °C above T_{ci} at 30 min, dropped to 0.8 °C soon thereafter, remained at this level over the following 2 h, then gradually abated over the next 1.5 h (fig. 2b). These responses to PFS and LPS were consistent with those in our previous studies [14]. CVF, which reduced the serum complement to below detection (fig. 1, panels 12–14), did not have any demonstrable effect on either of these responses (fig. 2a, b).

Experiment 2

The intravenous injection of PFS into the C3+/+ and C3–/– mice induced no demonstrable thermal effect other than that associated with the handling and the injection itself (fig. 3a). LPS challenge, on the other hand, caused a sustained 1 °C fever in both groups of mice (fig. 3b). These febrile responses were similar to those of the WT mice in experiment 1.

Experiment 3

There were no overall statistically significant differences between the thermal responses of CR3+/+ and CR3–/– mice to intravenously injected PFS, although the CR3–/– mice tended to recover more quickly from their stress hyperthermia than their CR3-sufficient counter-

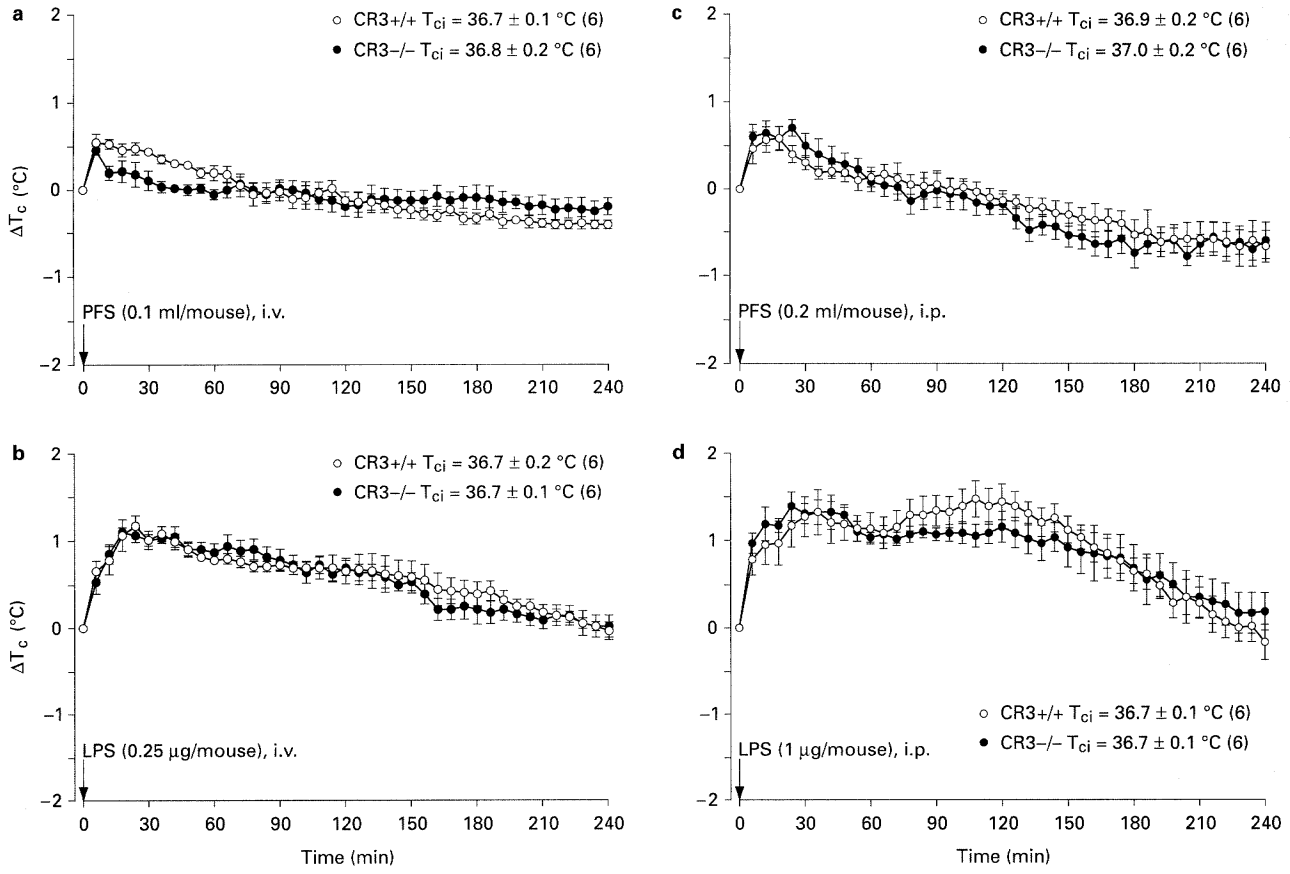

Fig. 4. Effects of intravenously or intraperitoneally injected PFS (0.1 ml, **a**; 0.2 ml, **c**) and *E. coli* LPS (0.25 µg/mouse, **b**; 1 µg/mouse, **d**) on the T_c of CR3+/+ and CR3–/– mice. Conditions and notations are as described in figure 2.

parts. Following the characteristic, transient, handling stress-induced hyperthermia, the T_cs of both groups of animals gradually returned toward their prehandling levels (fig. 4a). Both the CR3+/+ and CR3–/– mice developed febrile responses to intravenously injected LPS, the courses of which were not different from each other (fig. 4b). Similarly, except for some qualitative differences in patterns as compared with their responses to intravenously injected PFS and LPS, the thermal responses of both groups of mice to intraperitoneally administered PFS and LPS were not different from each other either (fig. 4c, d).

Experiment 4

The intravenous injection of PFS had no demonstrable thermal effect on either C5+/+ or C5–/– mice other than that associated with the handling and the injection itself (fig. 5a). C5+/+ mice developed a 1 °C fever after intravenous injection of LPS (fig. 5b) identical to that observed in the WT, C3+/+ and C3–/– mice. However, LPS did not cause T_c rises in the C5–/– mice other than those associated with the handling (fig. 5b), a response not different from that of the vehicle-treated animals (fig. 5a).

Experiment 5

The thermal responses of the WT and C3–/– mice that received PFS intravenously immediately after the intravenous injection of C5aRa or its vehicle, PBS, did not

differ significantly from each other (fig. 6a, c) or from those of their PFS-treated counterparts in experiments 1 (fig. 2a) and 2 (fig. 3a). In response to intravenously injected LPS, the PBS-pretreated mice also developed a fever similar in course and magnitude to those of the untreated (experiments 1 and 2) WT and C3–/– controls, respectively (figs. 6b, d). However, their C5aRa-pretreated counterparts did not develop a febrile response to intravenously injected LPS (fig. 6b, d).

The same, qualitative patterns of responses occurred when PFS or LPS was delivered intraperitoneally to the WT mice (fig. 7a, b); although, again, the courses of the T_c changes were somewhat different from those following the intravenous injections of those agents. The effects of C5aRa on the febrile response to intraperitoneal injection of LPS were not tested in the C3–/– mice.

Experiment 6

In this experiment, the initial T_cs of the C5+/+ and C5–/– mice, 30 min after the injection (see 'Methods'), were in the normal range, and the intracerebroventricular administration of PFS had no demonstrable thermal effect ($\Delta T_c < 0.5\,°C$; fig. 8). Intracerebroventricularly administered PGE_2, on the other hand, induced characteristic, very rapid rises in the T_cs of all the animals, i.e. when the recordings began, they were already greatly elevated in comparison with their PFS-treated T_cs or with their own recovery values, which they reached 90–120 min later (fig. 8a, b). There were no statistically significant differences between the febrile responses of the C5+/+ and C5–/– mice to intracerebroventricularly injected PGE_2. The T_cs of the LPS-treated C5+/+ mice 30 min after injection were approximately 0.5 °C higher than those of their PFS-treated counterparts. They then exhibited a slight, further rise (0.5 °C), remained at this level for 60 min, rose again, peaking at about 150 min (~1 °C above the T_cs at 30 min), and finally abated gradually to the level of their PFS-treated controls at about 330 min (fig. 8c). Except for an apparent difference in the duration to reach the peak, the courses of the fevers developed by the C5–/– mice after intracerebroventricular injection of LPS were similar to those of the C5+/+ mice (fig. 8d).

Experiment 7

The T_c responses of C5+/+ and C5–/– to intravenously injected PFS or LPS were similar to those in experiment 4, i.e. C5+/+, but not C5–/– mice developed a ~1 °C fever approximately 30 min after the intravenous LPS challenge (fig. 9a, c). Ninety minutes after LPS treatment, the serum levels of all three cytokines measured, IL-1β, TNFα and IL-6, of both the C5+/+ and C5–/– mice were significantly higher than those of their PFS-treated counterparts (fig. 9b, d). The respective levels of these cytokines, however, were not significantly different between C5+/+ and C5–/– mice.

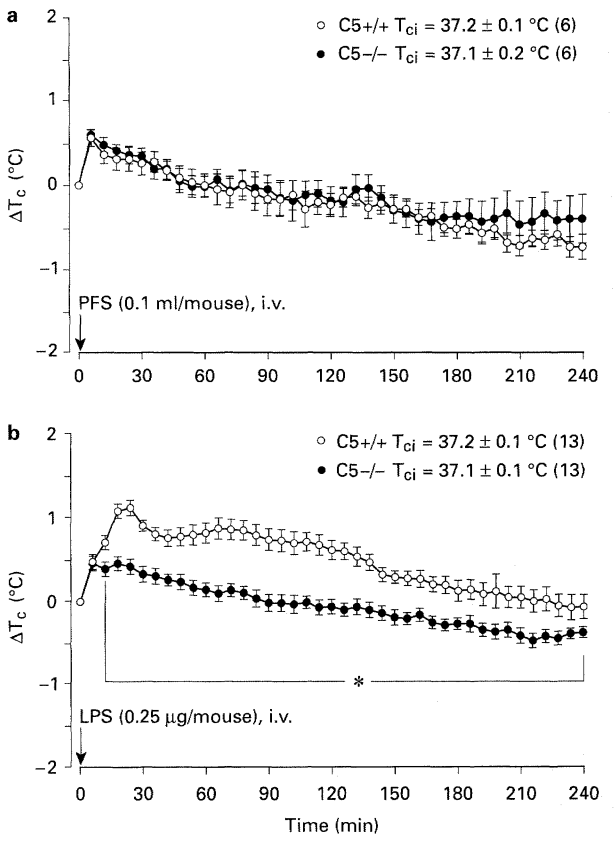

Fig. 5. Effects of intravenously injected PFS (0.1 ml; **a**) and *E. coli* LPS (0.25 µg/mouse in 0.2 ml of PFS; **b**) on the T_c of C5+/+ and C5–/– mice. Conditions and notations are as described in figure 2. * $p < 0.05$.

Discussion

The present results suggest C5a as the complement fragment most likely critically involved in the febrile response of mice to LPS, since the immediately prior administration of C5aRa prevented the ability of WT and C3–/– mice to develop a fever in response to an LPS challenge that induced a fever in their untreated counterparts,

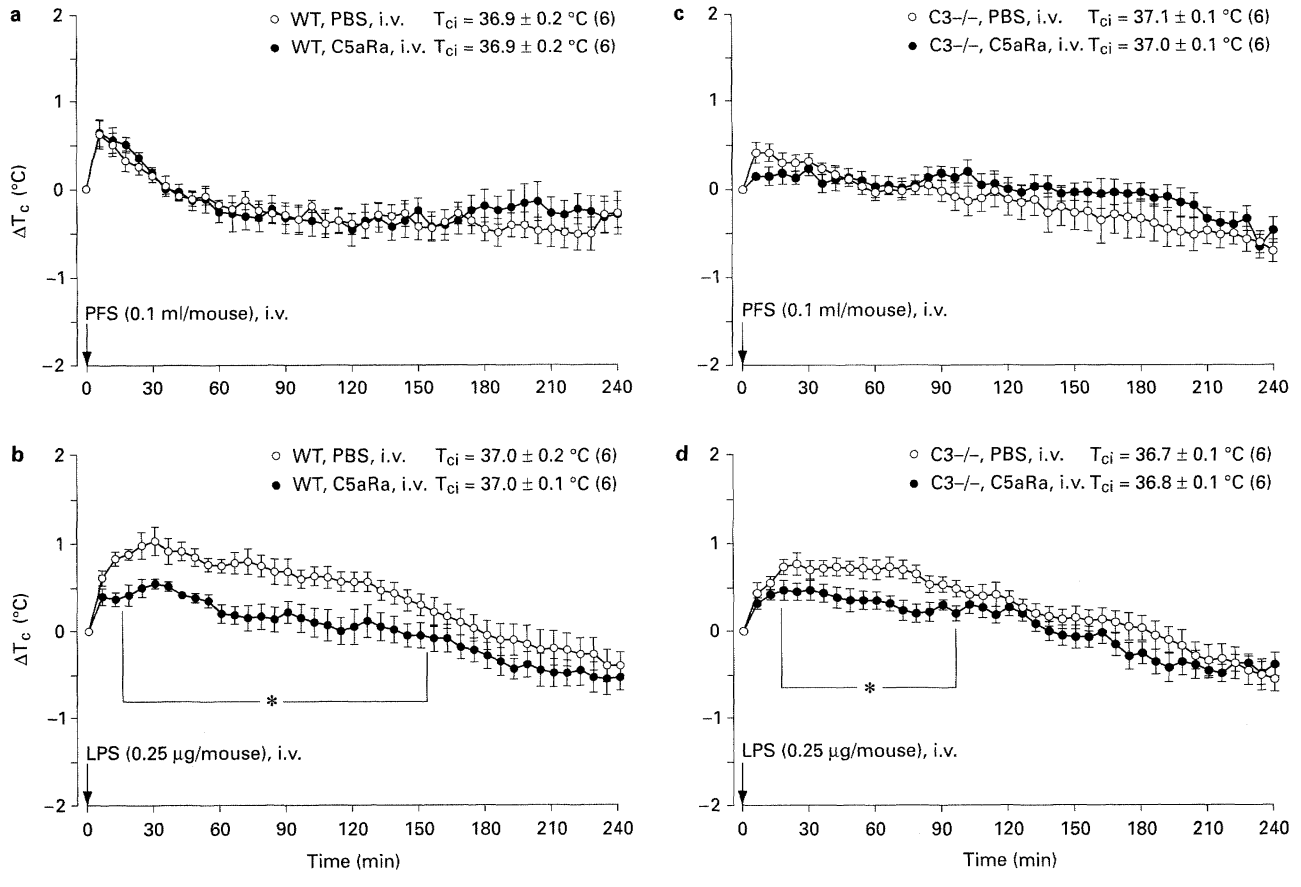

Fig. 6. Effects of intravenously injected PFS (0.1 ml; **a**, **c**) and *E. coli* LPS (0.25 µg/mouse; **b**, **d**) on the T_c of C57BL/6J (WT) and C3–/– mice pretreated intravenously with PBS (0.1 ml/mouse) or C5aRa (1 mg/kg in 0.1 ml of PBS). Conditions and notations are as described in figure 2. * $p < 0.05$.

irrespective of its route (intravenous or intraperitoneal) of administration (fig. 6, 7). This interpretation is further supported by the finding that the C5–/– mice were similarly unable to develop a fever in response to intravenously injected LPS (fig. 5), just as they were found earlier not to be able to respond to intraperitoneally injected LPS [14]. C3 does not seem to have a role in this response, since neither the febrile responses to intravenously administered LPS of the C3–/– (fig. 3) nor those of the CR3–/– mice (fig. 4) were affected as compared with those of their respective complement-sufficient controls. Furthermore, since the integrity of their downstream, central febrigenic controller was demonstrated in the C5–/– mice by the normal rise of their T_c after intracerebroventricular injection of PGE_2, and since these animals also developed normal febrile responses to intracerebroventricularly injected LPS, it may be concluded that immunomodulation of the febrile response to LPS by C5a occurs in the periphery rather than in the brain.

It is well documented that C5a is an important inflammatory mediator. It is, for example, a potent chemotactic agent for polymorphonuclear neutrophils, monocytes and other leukocytes, increases vascular permeability, stimulates a respiratory burst in polymorphonuclear neutrophils, induces the release of histamine from mast cells and basophils, and exerts other proinflammatory activities [for a review, see ref. 33]. Pertinent to the present study, C5a also activates myeloid and certain nonmyeloid cell types to release IL-1β, IL-6, TNFα and PGE_2 [34–42]; it also amplifies the secretions of IL-1β and TNFα induced

by LPS in these cells [11, 37]. C5a generates these products by interacting with C5a receptors (C5aR) expressed on these cells [43–46]. C5aR is normally expressed on Kc and hepatic stellate cells [9, 10, 47], and its expression in macrophages is significantly increased on stimulation by LPS [25, 48, 49]. The present finding that the blockade of C5aR by its antagonist (fig. 6, 7) prevents the development of fever in response to LPS indicates that C5a has a pivotal role in LPS-induced fever production and is congruous with other demonstrations that C5a inhibition by various means blocks several of its inflammatory actions [25, 50–52]. However, the specific mechanism and site of the pyrogenic action of C5a remain to be clarified. The possible involvement of C5b and/or its downstream assemblies in LPS-induced fever cannot yet be ruled out, however, since sublytic concentrations of the membrane attack complex, the ultimate product of the complement cascade, also recruit neutrophils and activate monocytes to release proinflammatory cytokines and chemokines [53, 54].

It is interesting that CR3 does not appear to be involved in the febrile response to LPS, since CR3–/– mice in this study responded to LPS, whether LPS was delivered intravenously or intraperitoneally, with T_c rises similar to those of their controls. CR3 (CD11b/CD18), a member of the β_2-integrin family expressed on monocytes and macrophages, is a receptor that binds both to unopsonized and iC3b-opsonized particles, such as LPS. It has been shown to be a signaling partner of CD14 and TLR4, the LPS/LBP receptors [55, 56], i.e. to act in concert with these receptors to elicit for example cytokines and COX-2 in response to a low concentration of LPS [56–60]. On the other hand, peripheral blood monocytes from CD18-deficient patients bind and respond to LPS normally [61], suggesting that CD14, which is expressed in CD18-deficient cells, can mediate LPS effects even when CD18 expression is profoundly depressed. On this latter basis, it would appear from our data that CD14 could also account for the febrile response to LPS of the present CR3–/– mice, and that CR3 has therefore no role in fever induction. In this regard, it has also been reported that CR3–/– mice exhibit significantly reduced numbers of peritoneal mast cells [62]. Extrapolating from this observation, it may be speculated from our findings, and in contrast to another recent report, that mast cells may not be material to the development of fever in response to an intraperitoneally injected LPS challenge either [63].

Our previous studies [12, 13] suggested that the complement system may be involved in the febrile response of guinea pigs to intraperitoneally injected LPS, because

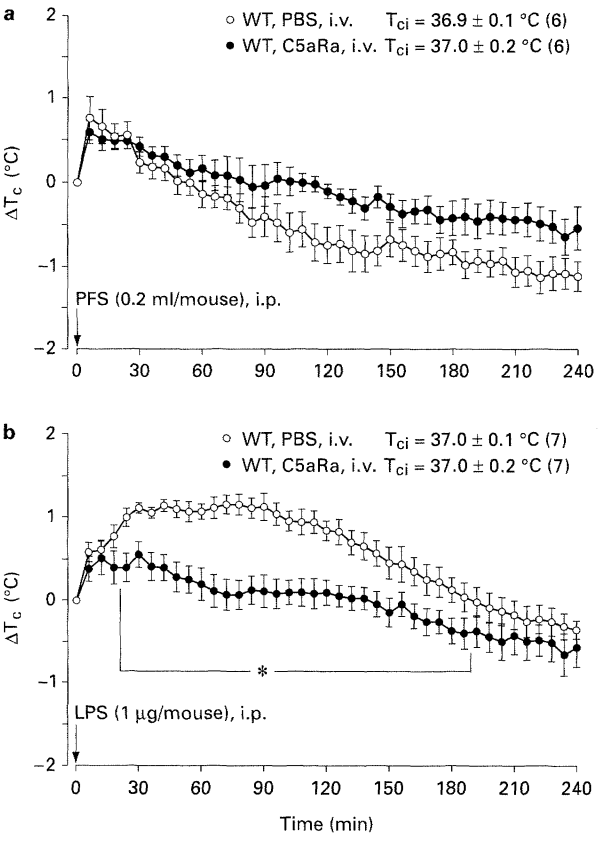

Fig. 7. Effects of intraperitoneally injected PFS (0.2 ml; **a**) and *E. coli* LPS (1 µg/mouse; **b**) on the T_c of C57BL/6J (WT) mice pretreated intravenously with PBS (0.1 ml/mouse) or C5aRa (1 mg/kg in 0.1 ml of PBS). Conditions and notations are as described in figure 2. * $p < 0.05$.

complement reduction by CVF dose-dependently attenuated the magnitude of their fever and eventually blocked their development altogether. The complement system, however, was apparently not involved when LPS was given intravenously, because guinea pigs whose serum complement levels were reduced to below detection by pretreatment with high doses of CVF still developed febrile responses similar to their vehicle-pretreated controls. Our more recent studies [14] confirmed the involvement of the complement system in the febrile response of WT (C57BL/6J) mice to intraperitoneally administered LPS, because CVF pretreatment also prevented their response, and because both C3–/– and C5–/– mice were not able to develop a fever after challenge with intraperitoneally administered LPS. On the presumption that C3–/– mice

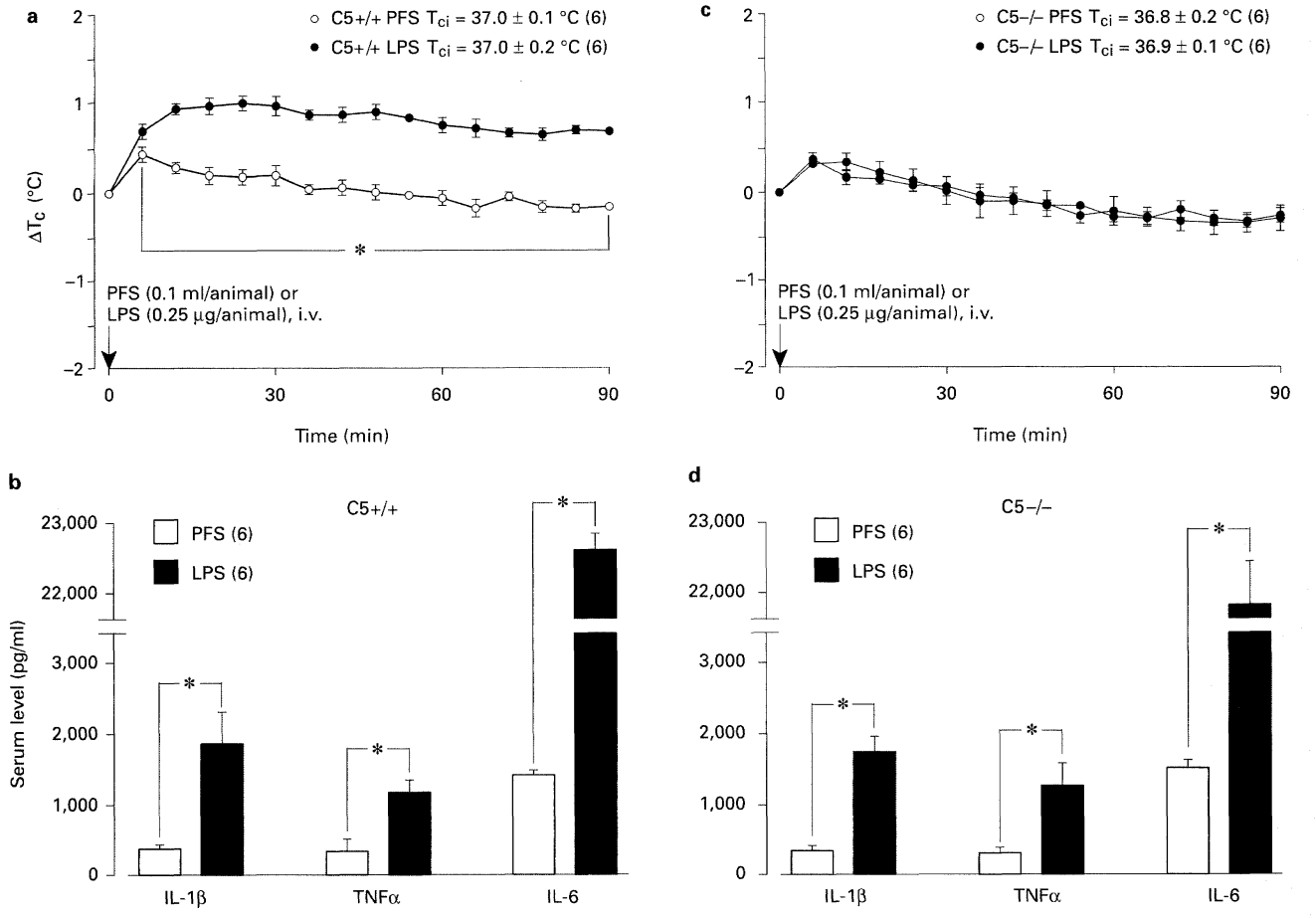

Fig. 9. Effects of intravenously injected PFS (0.1 ml/mouse) and *E. coli* LPS (0.25 μg/mouse) on the T_c and serum IL-1β, TNFα and IL-6 levels of C5+/+ (**a**, **b**) and C5–/– (**c**, **d**) mice. Serum samples were collected at 90 min after the PFS or LPS treatment. Other conditions and notations are as described in figure 2. * $p < 0.05$.

to C3bBb, the natural C3 convertase that catalytically cleaves the α-chain of C3. Therefore, the acute effect of CVF is to increase the production of C3b, C5 and their biologically active derivatives, including C5a. Since CVFBb is highly resistant to the normal control mechanisms that limit the activity of C3bBb, the fluid-phase complement activation continues unabated, drastically reducing C3. Consequently, absent from the substrate from which they are produced, all the subsequent complement components are also ultimately depleted. However, as we found in our earlier studies in guinea pigs, decomplementation was never complete [13]; at the highest CVF dose we used (200 U/guinea pig), complement was reduced by 95–98%. In this study, we used the maximum dose (10 U/mouse) that did not demonstrably affect the normal behavior of the mice. Thus, although total complement was apparently reduced to below detection (fig. 1), it is probable that some C5 remained that could have mediated the febrile responses of the present CVF-treated mice as well as of our guinea pigs earlier [13] to intravenously injected LPS.

(2) On the presumption that C3–/– mice should be unable to generate C5a and C5b (due to their inability to form the C5 convertase C3bBbC3b), the finding in this study that the C3–/– mice developed normal fevers in response to intravenously injected LPS was a priori unexpected. However, several papers have suggested that C5 could be generated in C3–/– mice by a pathway that does

not involve the participation of C3. Thus, serum-C3-like activity was found in dogs even when the antigenic C3 was less than 0.003% of the normal level [67]; the C5 level was about 57% of that in normal dogs. Another study showed that a small amount of C5 was activated in the serum of patients deficient in C3 by a C4/2 convertase complex which was able to activate C5 directly [68–71], resulting in the formation of C5a and of the membrane attack complex, C5b-9. Therefore, the presence in plasma of a small amount of C5 could have been sufficient to enable the febrile response of these C3–/– mice to intravenously injected LPS.

(3) We have shown recently that the onset of LPS-induced fever in guinea pigs is temporally related, irrespective of its routes of administration (intravenous or intraperitoneal), to the appearance of LPS in the liver and its uptake by Kc [72, 73], suggesting that the complement system may be activated by contact with LPS in the liver rather than in the bloodstream. This, in turn, suggests that C5 may be generated independently of a plasma-derived complement system, i.e. by extravascular components of the liver, e.g. by Kc. Indeed, synthesis of C5 by human and mouse macrophages has been described [74–78]. Macrophages also produce C5-cleaving serine proteases that can generate C5a [79–82], and Kc express C5aR [9, 10]. Thus, it is possible that C5a was generated in the present study by LPS-activated Kc from its autogenously produced C5 and functioned as an autocrine activator of Kc. Such a mechanism could also account for the observed febrile response of the C3–/– mice to intravenously injected LPS. On all these grounds, we surmise that the susceptibility of mice to the pyrogenic effects of intravenously and intraperitoneally injected LPS is not differentially affected by the absence of complement. For the time being, the unavailability of suitable, quantitative murine complement assays precludes, verifying the levels of C5 that may be extant in these various animals.

Recent studies showing that C5a and C5aR are also present under normal conditions in astrocytes, microglia and neurons of the brain [83–87] raise the possibility that the brain, which is the locus of the fever controller [18] and wherein fever-induced cytokines and PGE_2 are produced [20], could be the site of action of C5a. However, since the present C5–/– mice responded normally to intracerebroventricularly injected PGE_2 and developed fevers after intracerebroventricularly injected LPS identical to those of their C5+/+ counterparts, it would appear that the critical site of action of C5a is peripheral rather than central.

The possibility that the inability of C5–/– mice to develop a fever could be due to their inability to produce pyrogenic cytokines was contradicted by the present finding that both C5+/+ and C5–/– mice responded to intravenously injected LPS with similar increases in serum IL-1β, TNFα and IL-6 levels 90 min after the challenge, indicating that the complement system plays no role in conjunction with the production of pyrogenic cytokines in the febrile response to LPS.

In conclusion, the present results, taken together with our previous findings [14], indicate that C5a is a critical mediator of the febrile response of mice to LPS, whether administered intravenously or intraperitoneally, and that its site of action is peripheral. Its specific target cell(s) and the identity of the pyrogenic product(s) that may be consequentially released still remain to be clarified.

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