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Circulating Inflammatory Mediators during Start of Fever in Differential Diagnosis of Gram-Negative and Gram-Positive Infections in Leukopenic Rats

Eva Tavares,[1] Rosario Maldonado,[1] Maria L. Ojeda,[1] and Francisco J. Miñano[1,2]*

Unidad de Investigación, Laboratorio de Farmacología Clínica y Experimental, Hospital Universitario Virgen de Valme,[1] and Departamento de Farmacología, Radiología y Pediatría, Facultad de Medicina, Universidad de Sevilla,[2] Seville, Spain

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Gram-negative and gram-positive infections have been considered the most important causes of morbidity and mortality in patients with leukopenia following chemotherapy. However, discrimination between bacterial infections and harmless fever episodes is difficult. Because classical inflammatory signs of infection are often absent and fever is frequently the only sign of infection, the aim of this study was to assess the significance of serum interleukin-6 (IL-6), IL-10, macrophage inflammatory protein-2 (MIP-2), procalcitonin (PCT), and C-reactive protein (CRP) patterns in identifying bacterial infections during start of fever in normal and cyclophosphamide-treated (leukopenic) rats following an injection of lipopolysaccharide (LPS) or muramyl dipeptide (MDP) as a model for gram-negative and gram-positive bacterial infections. We found that, compared to normal rats, immunosuppressed animals exhibited significantly higher fevers and lesser production of all mediators, except IL-6, after toxin challenge. Moreover, compared to rats that received MDP, both groups of animals that received an equivalent dose of LPS showed significantly higher fevers and greater increase in serum cytokine levels. Furthermore, in contrast to those in immunocompetent rats, serum levels of IL-6 and MIP-2 were not significantly changed in leukopenic animals after MDP injection. Other serum markers such as PCT and CRP failed to discriminate between bacterial stimuli in both groups of animals. These results suggest that the use of the analyzed serum markers at an early stage of fever could give useful information for the clinician for excluding gram-negative from gram-positive infections.

Bloodstream infection is an important cause of morbidity and mortality, especially in febrile patients undergoing intensive chemotherapy for the treatment of cancer and other illnesses (10). This susceptibility appears to be directly related to the severity and length of leukopenia (28). Most infections in immunosuppressed patients are due to gram-negative and gram-positive bacteria (21), and fever is frequently the only sign of infection (7, 34). Thus, because in the majority of these patients with fever episodes the causative infectious agents cannot be identified due to the nonspecific nature of signs and symptoms (16), these patients are treated empirically with broad-spectrum antibiotics as soon as fever develops (6, 28). Therefore, because there are increasing numbers of patients who are immunodeficient following chemotherapy and because microbes are becoming antibiotic resistant, the use of inflammatory markers for early detection of bacterial infections could be essential to reflect the nature of the infection and distinguish the fever episodes in immunosuppressed patients in order to guide the choice of specific antibiotic therapies, even before culture results are available (27).

The systemic host responses to gram-negative and gram-positive bacterial infections involve similar clinical symptoms, including leukocytosis and fever (19, 44). Leukocytes, mainly neutrophils, are essential for resistance to bacterial infections, as they are the main source of proinflammatory mediators (25, 32). These cells express specific receptors for immunogenic bacterial cell wall constituents released during infection (1, 37).

Lipopolysaccharide (LPS) is believed to be the most important marker for gram-negative bacteria, whereas peptidoglycan by-products such as muramyl dipeptides (MDP) serve as salient stimuli from gram-positive bacteria (9, 19, 20, 44). Although gram-negative bacterial cell walls also contain peptidoglycan, its concentration is far greater in the walls of gram-positive bacteria (20, 44). Muramyl peptides are key elements of the immune response to gram-positive bacteria, which lack LPS. Presently, much work is focused on LPS effects, but many immunocompromised patients die of gram-positive infections (10). Immune cells activated by binding of these substances release many inflammatory mediators, including eicosanoids, cytokines, and chemokines, that activate other immune cells and can also serve to signal the brain.

In humans and experimental animals, MDP and LPS cause an induction of the acute-phase response, which is characterized by fever and the production of cytokines in vitro and in vivo (9, 20, 30), with cells and animals being more sensitive to LPS than to MDP (42). These studies indicate that the patterns of production of endogenous mediators involved in the primary nonspecific acute host response may be dependent upon

* Corresponding author. Mailing address: Departamento de Farmacología, Pediatría y Radiología, Facultad de Medicina, Universidad de Sevilla, 41009 Seville, Spain. Phone: 34-955-015877. Fax: 34-954-905970. E-mail: jminano@us.es.

the specificity of the microbial pathogens and the host recognition pathways invoked. Like for LPS, it is assumed that MDP produces fever via cytokines and prostaglandin E_2 induction (20, 44). The mechanisms underlying the febrile response to LPS and MDP in leukopenic animals were not elucidated in this study. This point remains to be resolved, although it is presently under investigation in our laboratory.

Although there are discrepancies regarding the involvement of specific receptors in recognition of gram-negative and gram-positive cell wall components (1, 38, 39, 43), LPS and MDP elicit similar fevers and apparently similar sickness behaviors. Much evidence now indicates that LPS and MDP activate different cell surface receptors and that the stimulation of these different receptors can lead to different pathways and profiles of proinflammatory mediators (13, 17, 18, 29). More recently, evidence has accumulated to indicate that, in contrast to the case for LPS, the response to MDP is CD14 independent and is recognized by nucleotide-binding oligomerization domain 2 but not by Toll-like receptor 2 (TLR2) or TLR4 MD-2 complex (14, 37).

Circulating proinflammatory mediators such as interleukin-6 (IL-6), IL-8, macrophage inflammatory protein-2 (MIP-2), C-reactive protein (CRP), and, more recently, procalcitonin (PCT) have been suggested to be predictive for a systemic microbial bloodstream infection in neutropenic hosts (4, 8, 11, 12, 24, 35, 36). However, the early cellular response to gram-negative and gram-positive bacteria is still unclear, and little is known about how the profile of these mediators during severe leukopenia might be affected by different microbial stimuli.

The present study was established to compare the induction of fever and proinflammatory mediators (IL-6, IL-10, MIP-2, CRP, and PCT) in normal and leukopenic rats following an injection of LPS or MDP as a model to discriminate between gram-negative and gram-positive infections. For this purpose, we used an experimental model that resembles the pathophysiologic events that may occur in patients receiving cyclophosphamide, an alkylating agent with cytotoxic and immunosuppressive activities used in the treatment of malignancies and as a combatant of transplant rejection.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male Wistar rats (Charles River Breeding Labs, Barcelona, Spain), weighing between 200 and 250 g, were used for the experiments. Rats were kept in a specific-pathogen-free facility and housed in individual plastic cages. All rats were maintained during all experiments in a temperature-, humidity-, and light-controlled chamber set at 26 ± 1°C with a 12-h, 12-h light-dark cycle, with light on at 0700 h. Rodent laboratory chow and drinking water were provided ad libitum. One rat was used for one experiment only and was not used repeatedly. Half of the rats were rendered leukopenic by intraperitoneal (i.p.) injections of cyclophosphamide before bacterial challenge, whereas the nonleukopenic group (normal, immunocompetent rats) did not receive any cyclophosphamide. All rats were acclimated for 1 week before the study. Experimental interventions were carried out with the rats remaining in their home cages. Care and use of the animals were in accordance with protocols approved by the Animal Ethics Committee of the University of Seville and were conducted according to the European Communities Council Directive (86/609/EEC).

Drug solutions. Cyclophosphamide, purified lipopolysaccharide from *Escherichia coli* (serotype 0111:B4), and MDP (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine) were obtained from Sigma Chemical Co. (St. Louis, MO). The vehicle for all solutions was sterile, pyrogen-free 0.9% saline (PFS) (Fresenius Kabi, Barcelona, Spain). The same lot and stock solutions were used for all experiments. All drug solutions were prepared in pyrogen-free glassware, passed through 0.22-μm-pore-size Millipore bacterial filters, warmed to 37°C, and briefly sonicated before i.p. administration. None of the MDP and cyclophosphamide solutions used in this study induced gelation in the *Limulus* amebocyte lysate assay, so any contamination with endotoxin was below the level of 25 pg/ml.

TABLE 1. Effect of cyclophosphamide on total leukocytes and differential cell counts in rats[a]

Cells	Count (10^9/liter)[b] in:	
	PFS-treated rats	Cyclophosphamide-treated rats
Total leukocytes	6.0 ± 0.3	0.1 ± 0.02*
Neutrophils	4.2 ± 0.3	0.06 ± 0.02*
Lymphocytes	1.5 ± 0.2	0.008 ± 0.001*
Monocytes	0.3 ± 0.1	0.01 ± 0.001*

[a] At 24 h after the second dose of cyclophosphamide or PFS, immediately before LPS or MDP treatment (day 0), numbers of total leukocytes, neutrophils, monocytes, and lymphocytes in the peripheral bloodstream were determined.

[b] Data represent the means (± SEM) for 10 rats in each treatment group. *, significantly different from the corresponding values in immunocompetent rats ($P < 0.05$ by ANOVA)

Body temperature measurement. Changes in core body temperature were measured every 5 min using remote radiobiotelemetry (Vitalview Series 4000 system; Mini Mitter, Sunriver, OR) with an accuracy of ±0.1°C. Briefly, anesthetized animals (100 mg/kg ketamine and 5 mg/kg xylazine, both administered by i.p. injection), were implanted i.p. with precalibrated temperature-sensitive radio transmitters (model PDT-4000 E-Mitters; Mini Mitter). Each animal was housed in a separate cage and allowed to recover for 1 week. The regular day-night body temperature rhythm was monitored for another week before the experiment. Animals remained in their home cages during the experiment, while the frequencies emitted by the thermistors were monitored remotely. These frequencies were then converted to temperature (°C) values based on each transmitter's calibration data. For the analysis and graphical documentation, temperature data from adequate time intervals of 15 min were used.

Induction of leukopenia. Chemotherapy-induced profound leukopenia in rats was achieved as described previously (24, 26). In brief, rats were rendered leukopenic by i.p. administration of cyclophosphamide at 150 and 50 mg/kg, 3 days and 1 day, respectively, before injection or any further treatment. This protocol resulted in leukocyte counts of $<0.1 \times 10^9$/liter on the day of drug injection (day 0), mainly due to a very low number of neutrophils. Also, the numbers of lymphocytes and monocytes are strongly reduced and contribute to the profound leukopenia observed in this experimental model (see Table 1). Animals were monitored for evidence of overt illness, activity, and body temperature after the treatments. No animals died following cyclophosphamide administration.

Experimental procedures. All experiments were conducted between 0700 and 1900 h. so that the circadian rhythms of the animals were identical across studies. Before the experiment, rats were weighed and arbitrarily assigned to body weight-matched experimental groups. Only animals whose body temperatures were stable and in the range of 36.9 to 37.3°C, which is within the range usually observed for rats under similar experimental conditions (23, 24), and which were acclimated to experimental procedures were used to determine the effect of drug application. All treatments were carried out at the same time of day, with each rat being used only once for the experiment. On the day of the experiment, the animals were separated into three treatment groups, namely, LPS, MDP, and PFS; each rat was handled gently so that the body temperature stabilized for a period of at least 90 min before any injection. For body temperature and hematological studies as well as the quantitative determination of inflammatory mediators, separate groups of normal and leukopenic rats were used in a time course experiment ($n = 10$ to 12 rats/group/time point).

Fever induction. Fever was induced by i.p. administration of a single dose of LPS (100 μg/kg) or MDP (100 μg/kg), as previously described (23, 30, 33, 41). Control animals received an equivalent volume of PFS as vehicle (1 ml/kg). The order of PFS and pyrogen injections was randomized. All LPS and MDP treatments were carried out 24 h after the second injection of cyclophosphamide, when leukocyte counts are very low (see Table 1). To avoid the development of toxin tolerance, each animal was injected with LPS or MDP only once. All injections were done at the same time of day (0700 h), so that the circadian rhythms of the animals were identical across studies. The changes in core body

temperatures were monitored for 12 h posttreatment. For hematological studies and the quantitative determination of inflammatory mediators, additional groups of normal and leukopenic rats were used. Blood samples were collected from rats anesthetized as described above via cardiac puncture (under terminal anesthesia) at selected times after drug injection for later measurement of cell counts and serum mediators.

Hematological parameters. For hematological studies, whole blood for cell counts was collected into sterile tubes containing K_3EDTA, and cells were counted by an automatic hematological analyzer equipped with veterinary software (Cell Dyne 3500; Abbot, Allentown, PA), which uses a laser beam and measures the light scattered by the cells to give the total and differential leukocyte counts. Total peripheral leukocytes, lymphocytes, monocytes, and neutrophils counts were determined at 1, 2, 4, 8, and 12 h after LPS, MDP, or PFS administration for both normal and cyclophosphamide-treated rats.

Inflammatory mediators. For measurement of IL-6, IL-10, MIP-2, CRP, and PCT production in serum, blood was collected into sterile tubes containing trisodium citrate and a proteinase inhibitor mixture (125 mmol/ml citrate, 5000 mmol/ml EDTA, 6000 mmol/ml *N*-ethylmaleimide, and 500 kIU/ml aprotinin). The blood samples were immediately centrifuged at 2,000 × *g* for 10 min at 4°C and stored at –80°C until the day of analysis (within 1 month). Quantitative determination of rat cytokines in serum was done by enzyme-linked immunosorbent assays, using specific kits for IL-6 (R&D Systems Europe, Abingdon, United Kingdom), IL-10 (IBL, Hamburg, Germany), and MIP-2 (BioSource International, Camarillo, CA). The lower detection limits were 10 pg/ml for IL-6, 5 pg/ml for IL-10, and 1 pg/ml for MIP-2. The serum PCT concentrations were measured using a chemiluminescent immunoassay kit (LUMItest PCT; B.R.A.H.M.S. Diagnostica GMBH, Berlin, Germany) with a lower detection limit of 0.1 ng/ml. CRP in serum was measured by a particle-enhanced immunoturbidometric method, using a Cobas Integra 700 (Roche Diagnostic System, Basel, Switzerland). All samples were analyzed in duplicate and were assayed at optimal concentrations, according to the manufacturer's instructions.

Data analysis. Data are presented as means ± standard errors of the means (SEM) and were analyzed using SigmaStat software (SPSS Inc., Chicago, IL). In graphs of the thermal and cytokine responses, the mean changes were plotted over time. Integrated areas under the curve (AUC), from 0 to 12 h posttreatment, were calculated by trapezoid analysis and were used to compare the differences in the responses of each rat to pyrogen and PFS injections. Data were analyzed by one-way analysis of variance (ANOVA). In cases where the comparisons using ANOVA were shown to be significant, a further test using Student-Newman-Keuls post hoc analyses was performed to compare the effect of each treatment. A P value of <0.05 was considered significant.

RESULTS

Induction of peripheral blood leukopenia by cyclophosphamide. As described before, i.p. administration of cyclophosphamide induced a severe leukopenia in rats. As shown in Table 1, the absolute leukocyte count (immunocompetent rats, 5,890 ± 350 cells/ml) was significantly reduced by cyclophosphamide (to 100 ± 20 cells/ml) during all experimental periods ($P < 0.05$), indicating a successful leukopenic model. This effect remains for 5 to 7 days (28, 32). Although the reduced number of blood neutrophils characterizes the present leukopenia (absolute neutrophil count, ≤60 cells/ml; $P < 0.05$), reduced numbers of lymphocytes (8 ± 1 cells/ml) and monocytes (10 ± 1 cells/ml) also contributed to the decreased total cell count ($P < 0.05$). Some characteristic toxic effects of immunosuppressive chemotherapy were noted; e.g., the leukopenic rats appeared more inactive and may have lost some weight. Figure 1 shows the effects of i.p. administration of 100 μg/kg LPS or 100 μg/kg MDP on the total count of white blood cells for immunocompetent and leukopenic rats. All immunocompetent rats had a transient leukopenia at 1 h after MDP or LPS administration ($P < 0.05$). This leukopenia was followed by leukocytosis due to a marked recovery in neutrophils to a level that exceeded the normal level at 4 or 8 h after MDP or LPS administration, respectively (data not shown). At 12 h, total peripheral leukocyte counts were similar to preinjection values (time zero) ($P > 0.05$). However, in contrast to the case for immunocompetent rats, the same doses of either LPS or MDP did not modify the leukocyte populations deteriorated from a normal range due to cyclophosphamide during all experimental periods ($P > 0.05$).

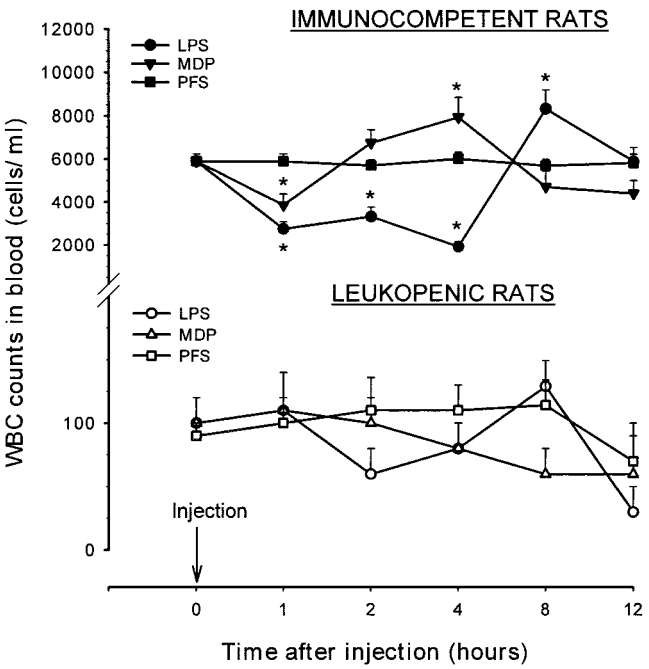

FIG. 1. Leukocyte (WBC) counts in peripheral bloodstreams of immunocompetent and leukopenic rats injected i.p. with 1 ml/kg PFS, 100 μg/kg LPS, or 100 μg/kg MDP. Leukopenia was induced by administration of cyclophosphamide 3 days (150 mg/kg) and 24 h (50 mg/kg) before PFS or pyrogen injection. The values presented are the means ± SEM for 10 to 12 rats per group. *, $P < 0.05$ (significantly different from the control values by one-way analysis of variance).

To determine the influence of peripheral leukocytes on body temperature and serum IL-6, IL-10, MIP-2, CRP, and PCT levels for distinguishing gram-negative from gram-positive bacterial infections, we serially measured the body temperature and the serum levels of these mediators in this model of leukopenic rats following i.p. administration of the gram-negative pyrogen LPS or the gram-positive pyrogen MDP.

Effects of LPS and MDP on body temperature. To test for the development of a pyrogenic response to LPS or MDP, normal rats and rats rendered leukopenic with cyclophosphamide were injected i.p. with 100 μg of either LPS or MDP per kg (Fig. 2). Importantly, the mean body temperature did not differ among the experimental groups, and all animals exhibited a similar basal body temperature that ranged between 36.9 and 37.3°C at the time of injection. The body temperature in the PFS-treated control groups remained stable throughout the study and was not different among groups ($P > 0.05$). Intraperitoneal injection of either LPS or MDP caused fever in both normal and leukopenic rats (Fig. 2A and B). Fifteen minutes after challenge with 100 μg LPS or 100 μg MDP, body temperatures in immunocompetent rats started to rise, and they reached the peak level at 6 to 8 h in normal rats (Fig. 2A). The return to the preinjection level was gradual but was es-

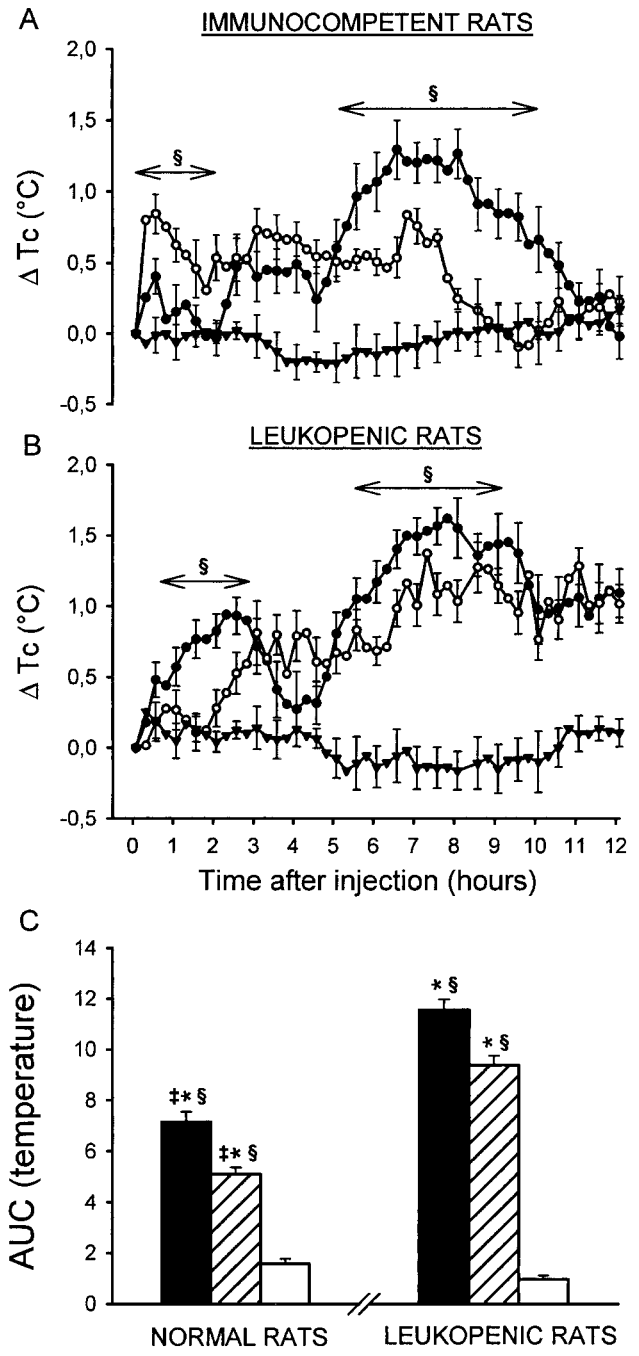

FIG. 2. Effect of LPS and MDP on body temperature in immunocompetent and leukopenic rats. Mean changes of core body temperature (ΔTc) were determined in separate groups of immunocompetent (A) and leukopenic (B) rats during 12 h after i.p. injection of either PFS (▼), 100 μg/kg LPS (●), or 100 μg/kg MDP (○). The mean baselines of absolute body temperatures at time zero for the groups were not significantly different (36.9 to 37.3°C). Integrated thermal responses (C) (AUC) of immunocompetent and leukopenic rats after administration of LPS (black bars), MDP (hatched bars), or PFS (white bars) were calculated for the whole 12-h recording period from the data shown in panels A and B (10 to 12 rats under each condition). Data are expressed as means ± SEM. § and ∗, statistically significant differences ($P < 0.05$) between LPS- and MDP-treated rats and normal rats, respectively. ‡, significantly different ($P < 0.05$) from corresponding values between immunocompetent and leukopenic rats (ANOVA, Student-Newman-Keuls post hoc test).

sentially completed by 12 h after challenge (Fig. 2A). In contrast, the fevers induced by LPS or MDP in leukopenic rats remained elevated up to 12 h following the injection and were significantly higher than those of the immunocompetent animals throughout ($P < 0.05$) (Fig. 2B). Thus, although the onset latencies of both groups of rats were not significantly different, the postfebrile recovery after LPS and MDP administration in leukopenic animals was not completed after 12 h after pyrogenic stimuli, and there was a significant enhancement in the fever (Fig. 2C). By 96 h postinjection, body temperature declined to or near to preinjection values (data not shown). As shown in Fig. 2C, injection of LPS or MDP in leukopenic rats resulted in a significant increase in core body temperature, which was significantly higher than that of the immunocompetent group (Fig. 2C). Furthermore, the febrile response evoked by LPS was significantly higher than that evoked by an equivalent dose of MDP in both groups of rats (Fig. 2C) ($P < 0.05$).

Effects of LPS and MDP on serum inflammatory mediators. In addition to measurement of core body temperatures, the kinetics of IL-6, IL-10, MIP-2, CRP, and PCT in response to LPS or MDP challenge were also studied. Significant differences were found between the kinetics of production of these mediators in normal and leukopenic rats, as well as in LPS- and MDP-treated rats. High secretion of IL-6, IL-10, MIP-2, PCT, and CRP was noted in sera of both immunocompetent and leukopenic rats after injection of a pyrogenic dose of either LPS or MDP (Fig. 3 and 4). We found for normal (nonleukopenic) rats that, in parallel with the body temperature change, the concentrations of IL-6 (Fig. 3A), IL-10 (Fig. 3C), MIP-2 (Fig. 3E), and CRP (Fig. 4C) in serum also started to rise at 1 h, reached the peak level at 2 to 4 h after LPS or MDP injection, and returned to the preinjection values at 12 h after challenge. However, unlike the case for these mediators, PCT started to rise at 2 h and reached its peak at 8 h after LPS or MDP administration (Fig. 4A), compared to the respective PFS controls. Furthermore, as shown in Fig. 5, LPS evoked a significantly higher production of serum mediators in both immunocompetent and leukopenic rats compared with the responses evoked by an equivalent dose of MDP ($P < 0.05$).

In leukopenic rats, the concentrations of IL-6 (Fig. 3B), IL-10 (Fig. 3D), and MIP-2 (Fig. 3F) in serum also started to rise at 1 h after LPS challenge. However, in contrast to those in immunocompetent rats, serum IL-6 and MIP-2 levels in rats treated with MDP did not differ from those in leukopenic rats treated with their respective PFS controls at any time point. Compared to leukopenic animals, the peak levels of all mediators, except for IL-6, were significantly higher in immunocompetent rats (Fig. 5) ($P < 0.05$). Similar to the case for immunocompetent rats, PCT and CRP failed to discriminate between bacterial stimuli in leukopenic rats (Fig. 4B and D).

The comparative study using analysis of integrated areas under the curve during the early phase of LPS- or MDP-induced fever showed significant differences between immunocompetent and leukopenic rats (Fig. 5). Baseline levels of IL-6, CRP, and PCT in serum were significantly higher in leukopenic rats. In contrast, baseline levels of MIP-2 were significantly higher in immunocompetent animals, and those of IL-10 were not detectable in either group of rats. Injection of LPS or MDP evoked a significant decrease in IL-10, MIP-2, and PCT levels in leukopenic rats compared to immunocompetent rats ($P <$

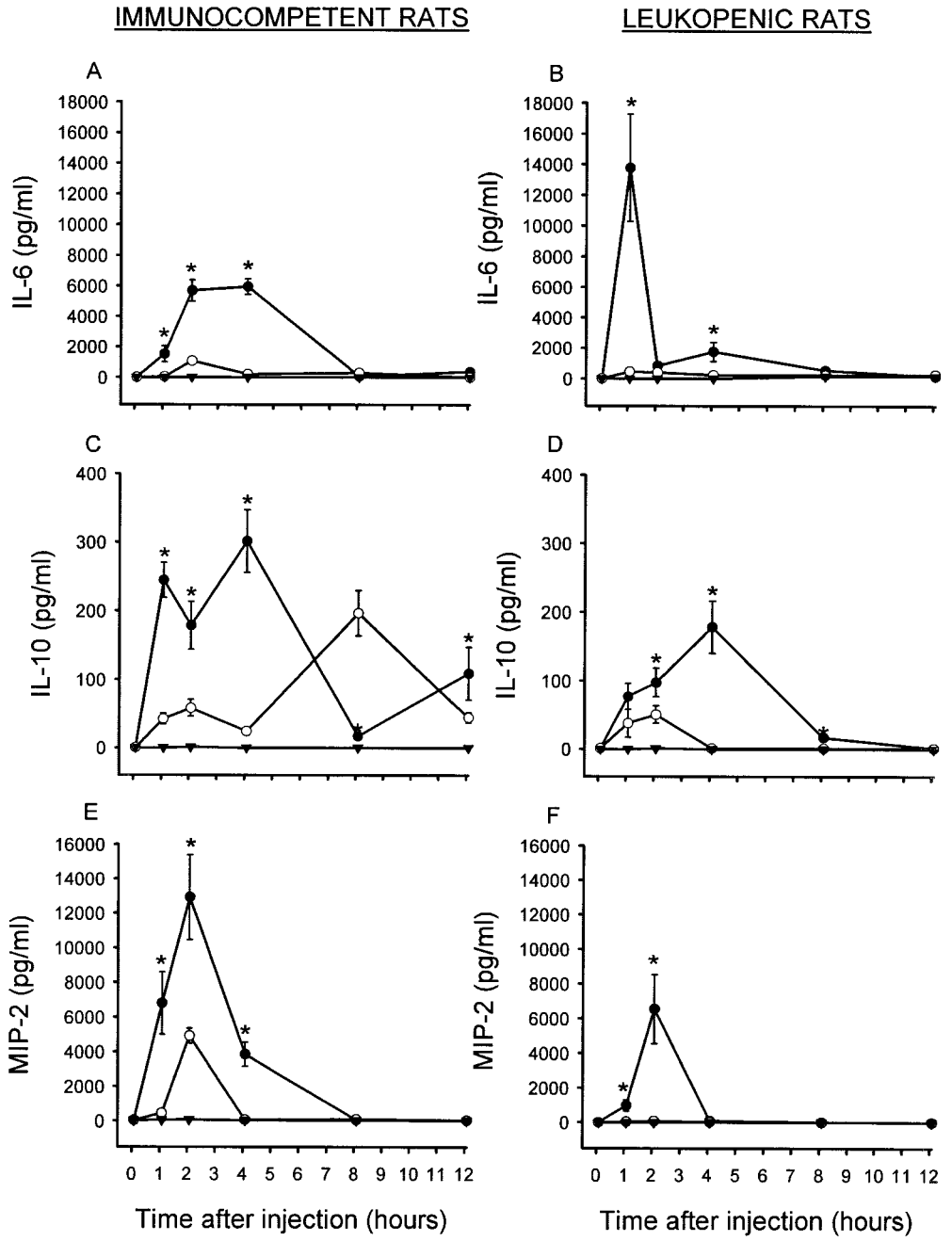

FIG. 3. Effects of LPS and MDP on serum IL-6, IL-10, and MIP-2 levels in immunocompetent and leukopenic rats. Serum levels of IL-6 (A and B), IL-10 (C and D), and MIP-2 (E and F) in separate groups of immunocompetent and leukopenic rats were determined at 1, 2, 4, 8, and 12 h after i.p. injection of either PFS (▼), 100 μg/kg LPS (●), or 100 μg/kg MDP (○). Each experiment involved 10 to 12 rats per time point. At all time points tested after injection of PFS (control), immunocompetent and leukopenic rats showed no significant elevation in serum levels of these cytokines ($P > 0.05$). Symbols represent means; bars indicate SEM. *, $P < 0.05$ between LPS- and MDP-injected rats (ANOVA, Student-Newman-Keuls post hoc test) for the same time point.

0.05) (Fig. 5). By contrast, IL-6 production was significantly higher in leukopenic rats than in immunocompetent rats after LPS administration ($P < 0.05$) but not after MDP administration ($P > 0.05$). Both groups of rats that received MDP showed lesser increases in serum IL-6 (Fig. 5A), IL-10 (Fig. 5B), and MIP-2 (Fig. 5C) production than were observed in rats that received a similar dose of LPS. Finally, PCT and CRP failed to discriminate between bacterial stimuli in both groups of animals (Fig. 5D and E).

DISCUSSION

The present findings indicate that cytokines such as IL-6, IL-10, and MIP-2 are rapid and sensitive markers to differen-

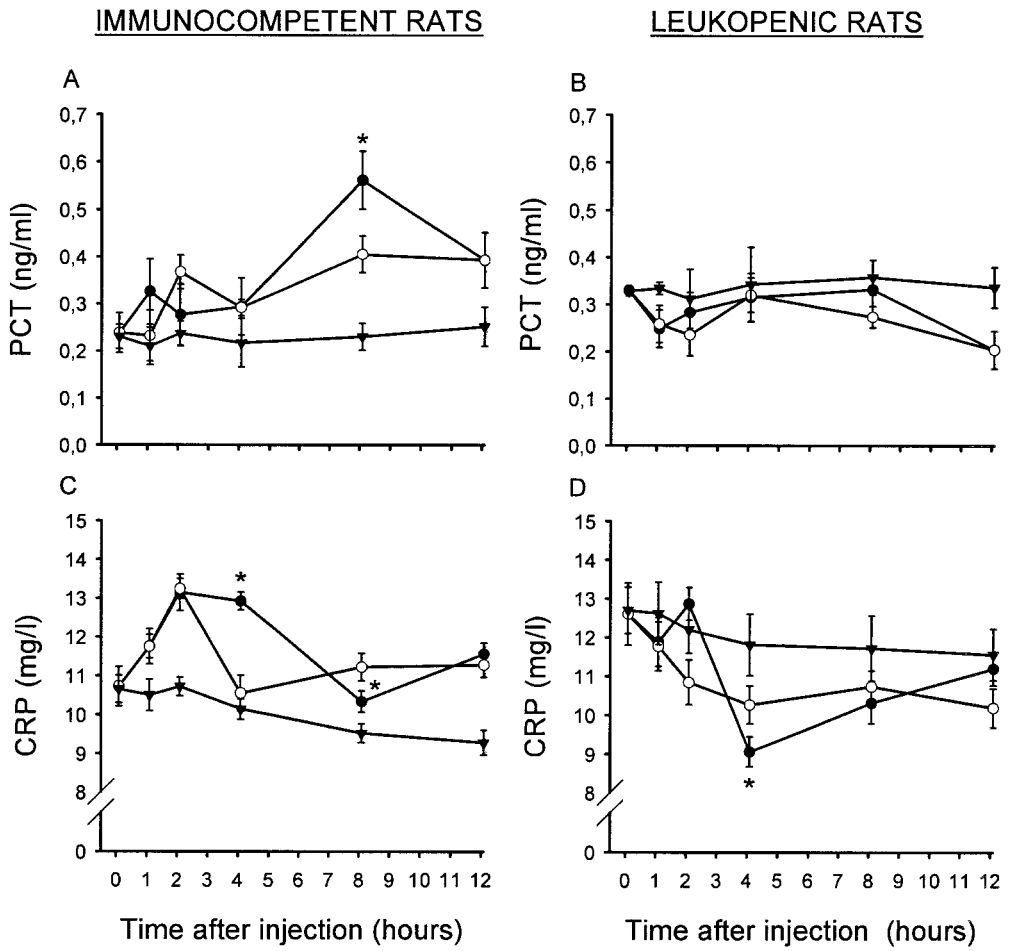

FIG. 4. Effects of LPS and MDP on serum CRP and PCT levels in immunocompetent and leukopenic rats. Serum levels of CRP (A and B) and PCT (C and D) in separate groups of immunocompetent and leukopenic rats were determined at 1, 2, 4, 8, and 12 h after i.p. injection of either PFS (▼), 100 μg/kg LPS (●), or 100 μg/kg MDP (○). Each experiment involved 10 to 12 rats per time point. At all time points tested after injection of PFS, both immunocompetent and leukopenic rats showed no significant elevation in serum levels of these mediators ($P > 0.05$). Symbols represent means; bars indicate SEM. *, $P < 0.05$ between LPS- and MDP-injected rats (ANOVA, Student-Newman-Keuls post hoc test) for the same time point.

tiate gram-negative from gram-positive stimuli in immunocompetent and, especially, in leukopenic rats when measured at an early stage of fever induced by specific pyrogens, such as LPS and MDP. Our results further suggest that CRP and PCT are not specific markers to discriminate between these bacterial infections. We have chosen these inflammatory markers because of interrelationships in the primary nonspecific host response to microbial infection (11, 12, 17, 18). The levels of other cytokines, such as IL-1 or tumor necrosis factor, were not determined in the present study because of poorer predictive value for the presence of bloodstream infection in patients with fever (15).

Gram-negative and gram-positive organisms have been considered the most important causes of infection in leukopenic patients who undergo chemotherapy. It is therefore not surprising that bacterial constituents that are present on the outside of the cell and therefore readily accessible to detection have been selected by the immune system as indicators of bacterial presence and potent inducers of a host response. During a bacterial infection in neutropenic patients, it is of vital importance to the infected host that the presence of pathogenic bacteria is detected as soon as possible, since these patients present a high morbidity and this may lead to mortality if the infection is treated inappropriately or too late. The present knowledge regarding endogenous inflammatory mediators in leukopenic patients is limited but indicates that the host response to microbial infection is mediated by the release of some pyrogenic mediators into the bloodstream (see the introduction).

Studies with immunocompromised patients have shown that these patients are capable of producing high serum concentrations of several mediators during severe systemic bacterial infection (4, 11, 21). In febrile neutropenic patients, elevated circulating IL-6 and IL-8 also predicted microbial infection (8, 12). In some reports, significant differences in the serum levels of different inflammatory mediators between gram-negative and gram-positive infections were described. Several investigators found differences between neutropenic patients with and without bacteremia and/or differences between gram-negative and gram-positive bacteremia. The latter observations, how-

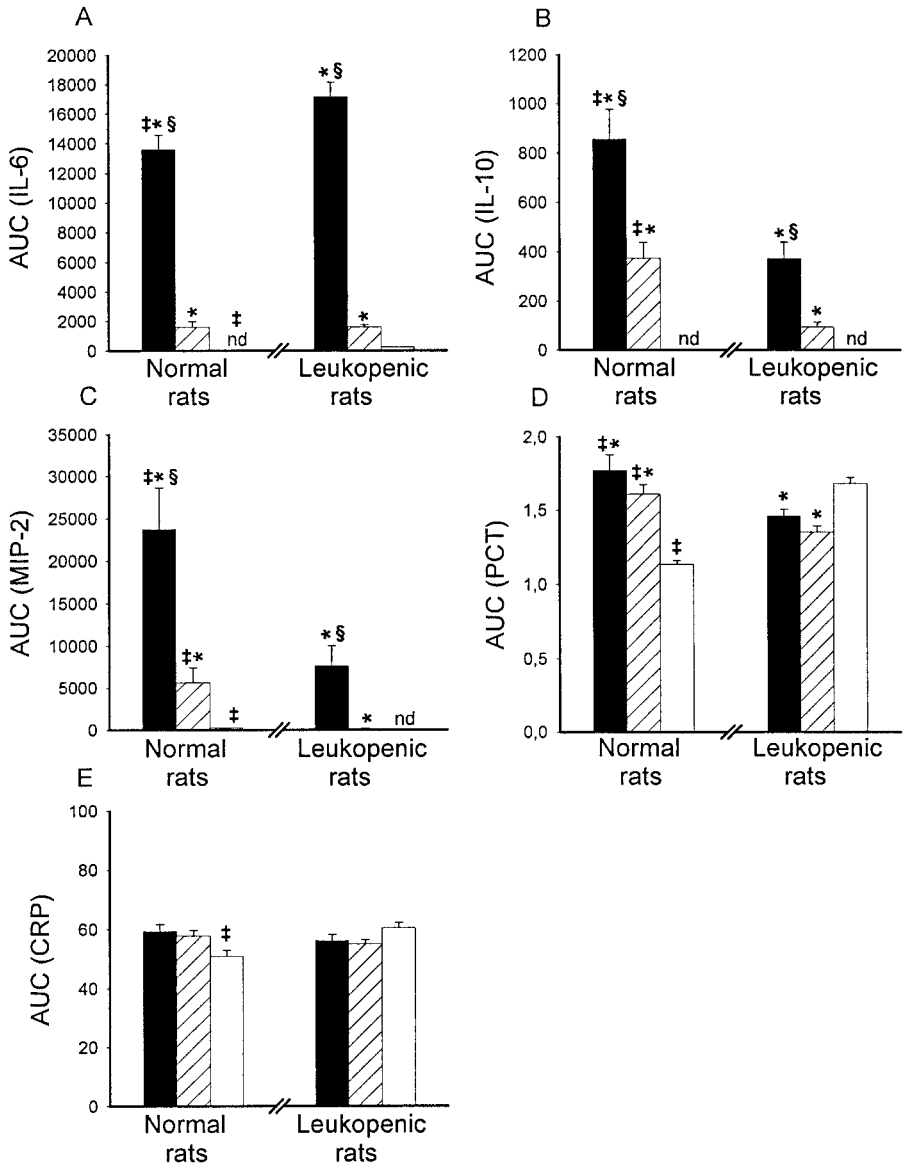

FIG. 5. Mean integrated IL-6 (A), IL-10 (B), MIP-2 (C), CRP (D), and PCT (E) responses of immunocompetent and leukopenic rats after PFS (white bars), LPS (black bars), or MDP (hatched bars) challenge. Statistical analysis was performed on AUC data from 0 to 12 h after the time of injection and was calculated from the data shown in Fig. 3 and 4. Bars represent means ± SEM for each group of rats (nd, not detected). § and *, statistically significant differences ($P < 0.05$) between LPS-and MDP-treated rats and their respective PFS-treated groups, respectively. ‡, significantly different ($P < 0.05$) from corresponding values between immunocompetent and leukopenic rats (ANOVA, Student-Newman-Keuls post hoc test).

ever, are difficult to interpret. Many patients with other causes of fever are omitted from such an analysis, but they need to be considered in order to calculate true sensitivities and specificities.

A key event in the initiation of fever in response to acute bacterial infection is the induction and release of endogenous pyrogens (i.e., IL-1, IL-6, IL-8, RANTES, and MIPs) by polymorphonuclear and mononuclear leukocytes into the bloodstream (19, 23, 32, 40, 41, 44). Normal leukocytes contain no detectable pyrogen and do not produce any during in vitro incubation. After stimulation, however, pyrogen production begins within a few hours and continues at a steady rate for 12 h or more (5). The key event is a rapid activation of humoral cascade systems and of leukocytes that support the inflammatory reaction. These cells are thought to be an essential target for gram-positive and gram-negative bacteria, as they are the main source of inflammatory mediators involved in the host acute response to these organisms (32). We found that leukopenic rats exhibit higher fevers after systemic injection of LPS and MDP than immunocompetent rats, despite lower increase in circulating mediators. Moreover, we found that, independently of the immune status, rats that received similar doses of LPS showed higher fevers and greater increases in serum cytokine levels than those observed after MDP challenge. Inter-

estingly, febrile responses actually subsided before the disappearance of elevated levels of serum cytokines after injection of LPS and MDP in leukopenic rats. These results are consistent with the idea that even when cytokines are detected in the blood, their timing does not always correlate with acute-phase activation after administration of immune stimuli (19).

Concerning the role of administration of cyclophosphamide, it has previously been shown that this immunosuppressive drug induces a nonspecific leukopenia (fewer than 100 cells/ml for 6 to 7 days) (24, 26) and a selective depletion of the lymphoid tissue (2, 42). When such treated patients become afflicted with a bacterial infection, they develop high fevers. These results suggest that a lesser contribution of leukocytes may be important to the exaggerated fever that occurs in leukopenic patients. However, in addition to peripheral blood leukocytes, the tissue resident macrophages are required for host defense against acute bacterial infection. In this context, production of cytokines at the tissue level, rather than in circulation, could be an alternative pathway for the induction of the signal leading to fever in leukopenic animals.

Because the liver is the principal organ for LPS clearance and its resident macrophages, the Kupffer cells, are a major source of pro- and anti-inflammatory cytokines, it has been suggested that these cells could be central to production of fever by LPS (44). However, the activity of Kupffer cells is also depressed by cyclophosphamide in mice and rats (2, 22), and temporary blockade of these cells with gadolinium chloride, a substance known to eliminate Kupffer cells (31), did not prevent the febrile response to i.p. LPS in leukopenic rats (E. Tavares, unpublished data). These results indicated that other cells types (fibroblasts, endothelial cells, glial cells, etc) involved in the acute inflammatory response are major sites of the action of bacterial toxins in immunosuppressed rats.

The present results indicate that cytokines such as IL-6, IL-10, and MIP-2 are regulated or released independently of the leukocyte count. How the absence of leukocytes leads to an intensified rather than a reduced febrile response to LPS and MDP and an associated decreased production of circulating inflammatory mediators is still unknowable. That is, the reduction of circulating leukocytes by cyclophosphamide caused significantly enhanced fevers (in both magnitude and duration), and this effect was associated with a significant reduction of circulating inflammatory cytokines compared to those in immunocompetent animals. The present findings confirm previous experimental and clinical studies that show that the production of fever was not dependent on the presence of leukocytes in the peripheral circulation (3, 24, 28). Furthermore, they suggest that other, yet-to-be identified mediators as possible mechanisms of immune signaling are involved in the development of the acute-phase response to gram-negative and gram-positive infections. Together, these findings suggest that in immunosuppressed animals leukocytes may play a "negative" role in the development of a fever induced by exogenous pyrogens. However, further studies on the pathophysiology of fever and the contribution of early markers regulated or released independently of the leukocyte count are necessary to verify this hypothesis.

Our findings suggesting that the patterns of early production of proinflammatory mediators may be dependent upon the specificity of the microbial pathogens are in agreement with recent studies (13). The differences in the cytokine pattern and the febrile responses to LPS and MDP may correspond to the different cell surface receptors stimulated by these bacterial pyrogens, with leukopenic animals being more sensitive to LPS than to MDP, which is in agreement with previous in vitro and in vivo studies (43). Functional characterization of TLRs has established that innate immunity is a skillful system that detects invasion of microbial pathogens. Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct development of antigen-specific acquired immunity (37). In agreement with these findings, the present results indicate that the patterns of production of endogenous mediators involved in the primary nonspecific acute host response may be dependent upon the specificity of the microbial pathogens and the host recognition pathways invoked.

The present findings also illustrate the potential differences in the patterns of whole-blood leukocytes in response to two disparate, but common, bacterial stimulants. Taking all these data together, it is improbable that the pyrogenic effects of LPS and MDP seen clinically in leukopenic patients are directly and exclusively due to stimulation of pyrogen production by leukocytes. Overall, serum IL-6, IL-10, and MIP-2 levels are able to discriminate between gram-negative and gram-positive infection at start of fever in both immunocompetent and leukopenic rats with acceptable specificity. Furthermore, they suggest that the exaggerated fever response occurring after LPS injection in leukopenic animals is partially mediated via IL-6. On the other hand, CRP and PCT are not sensitive markers in distinguishing these types of infections. However, although we used an experimental rat model that resembles the pathophysiologic events that may occur in leukopenic patients, the findings reported here cannot necessarily be extrapolated to the response to different gram-negative or gram-positive pathogens or their constituents in immunosuppressed patients. The mechanisms underlying the febrile response to LPS and MDP in leukopenic animals were not elucidated in this study. This point remains to be resolved, although it is presently under investigation in our laboratory.

The data presented here extend the argument that LPS and MDP induce differential fever responses and cytokine profiles in normal and immunodeficient animals. Finally, these results indicate that the determination of the serum pattern of specific inflammatory markers at the onset of fever may be used as an early diagnostic tool to differentiate gram-negative from gram-positive infections in both immunocompetent and leukopenic hosts. A rapid bedside test may thus help guide the choice of specific antibiotic therapies, especially for neutropenic patients, even before culture results are available.

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