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A Protective Role of Platelet-Activating Factor in Murine Candidiasis

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Platelet-activating factor (PAF) is a potent phospholipid-derived modulator of immunological and inflammatory processes. In this study, the role of exogenous and endogenous PAF in resistance to infection with *Candida albicans* was investigated. Administration of PAF following a lethal challenge of *C. albicans* significantly protected mice from death and reduced the number of organisms in the kidneys. Neutralization of endogenous PAF with the PAF antagonist BN50739 shortened the mean survival time and increased the number of *C. albicans* cells per kidney. Shortly after infection of mice (30 min), significant levels of PAF were detected in the serum. PAF-induced protection appears to be mediated through the actions of tumor necrosis factor alpha (TNF-α), since pretreatment with anti-TNF-α before each injection of PAF abrogated the majority of PAF-induced enhanced resistance. Administration of PAF in vivo elevated serum TNF-α levels and TNF-α mRNA expression in the kidney. Production of TNF-α was markedly diminished by pretreatment with the PAF antagonist BN50739 prior to infection with *C. albicans*. We conclude that PAF, which is produced during infection with *C. albicans*, plays an important role in determining the level of resistance to this infectious microorganism. This effect of PAF appears to be mediated, at least in part, through the induction of TNF-α.

Candida albicans, a part of the normal flora of mucosal surfaces of most healthy individuals, is a dimorphic opportunistic fungus which can cause a fatal systemic infection in an immunocompromised host (8, 31). The precise mechanism utilized by the immune system to control the growth of *Candida* is not well understood. It is known, however, that both phagocytic cells (29, 55) and specific cell-mediated immune responses (3, 30) participate in host defenses against this organism.

Evidence has accumulated that the various cytokines produced by T cells and inflammatory cells contribute to the host response against *Candida* (see reference 4 for a review). Tumor necrosis factor alpha (TNF-α) (35, 51), interleukin-1 (IL-1) (33, 43), and gamma interferon (32, 46) have been shown to enhance the resistance of mice to systemic *C. albicans* infection, whereas the type 2 cytokines IL-4 (45, 48) and IL-10 (49) diminish resistance.

Platelet-activating factor (1-*o*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; PAF) is produced by a variety of cells involved in inflammatory reactions, including neutrophils, basophils, eosinophils, monocytes/macrophages, platelets, and endothelial cells (12, 15, 36). PAF has been implicated in a number of diverse pathological conditions, including shock, allergic reactions, thrombosis, and a variety of inflammatory diseases (9). Recently, it has been reported that PAF and proinflammatory cytokines such as TNF-α and IL-1 stimulate the release of each other (11, 13, 20, 26, 37, 54). PAF is released in response to TNF-α or IL-1 (11, 13, 54); in turn, PAF mediates and/or enhances the release of these cytokines (20). Furthermore, recent studies performed by us (26) and others (37) demonstrated that the inhibition of PAF activities via a PAF antagonist reduces the ability of cells to produce TNF-α in response to an inflammatory stimulus. Therefore, based on these observations, it is of interest to investigate whether PAF has any regulatory effect on host resistance to candidiasis.

In this study, we demonstrate that PAF has a protective role in systemic murine candidal infection and that this effect of PAF appears to be mediated by TNF-α.

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MATERIALS AND METHODS

C. albicans. *C. albicans* NIH A-207 was kindly provided by Hideoki Ogawa (University of Juntendo, Juntendo, Japan). *C. albicans* was grown to stationary phase at 28°C under slight agitation in Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, Md.). After a 24-h culture, cells were harvested by centrifugation (2,000 × *g*), washed twice in phosphate-buffered saline (PBS), diluted to the desired density, and injected intravenously (i.v.) via the tail vein in a volume of 0.2 ml.

Mice. Specific-pathogen-free female BALB/c mice were purchased from the Korean Research Institute of Chemistry Technology (Daejeon, Korea). They were housed throughout the experiments in a laminar-flow cabinet and maintained on standard laboratory chow ad libitium. All mice were used at 7 to 8 weeks of age.

Reagents. PAF was purchased from Sigma Chemical Co. (St. Louis, Mo.). The PAF antagonist BN50739 (batch 51-884; M_r, 596.2; 50 mg/ml in dimethyl sulfoxide), a ginkoglide-derived synthetic PAF analog, was a gift from Pierre Braquet (Institute Henri Beaufour, Le Plessis Robinson, France) and was stored at −20°C. Murine TNF-α was purchased from R & D Systems (Minneapolis, Minn.).

Quantification of *C. albicans* in kidneys. Kidneys from individual mice were removed aseptically and homogenized with 5 ml of PBS. The number of viable CFU in the specimens was determined by dilution plating on Sabouraud dextrose agar (BBL).

Pretreatment of anti-TNF-α antibody. Polyclonal anti-murine TNF-α antibody was prepared from a New Zealand White rabbit. The rabbit was immunized by multiple subcutaneous injections to the shaven back with 50 μg of TNF-α mixed 1:1 with complete Freund's adjuvant (Sigma) (total volume, 1 ml). Booster injections of 25 μg of TNF-α mixed with incomplete Freund's adjuvant (Sigma) were given 3, 6, and 9 weeks later. Total bleeding was performed 3 weeks after the last injection, at which time the titer of the serum was 1:12,000 in an enzyme-linked immunosorbent assay (ELISA) in which microtiter plates were coated with 5 μg of TNF-α per ml. The serum preparation was precipitated in 45% ammonium sulfate and dialyzed against pyrogen-free distilled water, and the immunoglobulin G (IgG) fraction was purified by protein G chromatography (Pierce Chemical Co., Rockford, Ill.). The protein content was determined by using a protein assay from Bio-Rad (Hercules, Calif.). Lipopolysaccharide content (*Limulus* amoebocyte lysate assay) was less than 9.4 pg/mg of protein. The

control IgG fraction was prepared by using the same procedures on preimmune serum. The activity of the anti-TNF-α antibody was determined by an L929 bioassay (see below). Each milligram of antibody neutralized 500 μg of TNF-α. Control IgG from preimmune serum did not show any neutralizing activity.

L929 TNF bioassay. The L929 assay was performed as previously described (58). Briefly, serial twofold dilutions of TNF-α were added to L929 cells cultured at a density of 2×10^4/well in the presence of actinomycin D (1 μg/ml; Sigma). After 18 h at 37°C and 5% CO_2 in air at 90% relative humidity, TNF-mediated cytopathic effects on L929 cells were evaluated spectrophotometrically at 540 nm following the addition of crystal violet. The detection limit of the assay was 100 pg/ml.

Measurement of plasma PAF. Blood was collected by cardiac puncture under ether anesthesia. Plasma PAF was quantified as described previously (26), using SI silica column chromatography (Amersham International Plc., Buckinghamshire, England) and an SPRIA kit (Amersham).

Measurement of serum TNF-α. Serum TNF-α was quantified by using an ELISA kit from Endogen (Boston, Mass.) as instructed by the manufacturer.

Reverse transcriptase PCR. RNA was prepared as previously described (17). Reverse transcription was performed with 1 μl of total RNA in a 10-μl reaction mixture (Promega, Madison, Wis.) containing oligo$(dT)_{15}$ and avian myeloblastosis virus reverse transcriptase. cDNA (1 μl) was amplified by PCR in a thermal cycler (Molecular Biology Technique Center [Kent, United Kingdom] ThermojeT) (denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and elongation for 40 s at 72°C), using TNF-α (30 cycles) or β-actin (23 cycles) primers. The primers used in these analyses are as follows: β-actin, 5′GGG TCA GAA CTC CTA TG3′ and 5′GTA ACA ATG CCA TGT TCA AT3′; and TNF-α, 5′CCT GTA GCC CAC GTC GTA GC and 5′TTG ACC TCA GCG CTG AGT TG3′.

Statistical analysis. The data are presented as the means ± standard errors of multiple determinations from an experiment. Statistical significance was determined by Student's *t* test when two data sets were analyzed or, alternatively, by analysis of variance followed by the appropriate post hoc test for multiple data sets with the statistical software Staview (version 4.01). All experiments were conducted two or more times. Reproducible results were obtained, and representative data are shown in the figures.

RESULTS

Effect of PAF on survival and growth of *C. albicans*. We investigated whether exogenous PAF could influence host resistance to *C. albicans*. As shown in Fig. 1A, PAF exerted a profound protective activity in a time-dependent manner. All of the control animals died within 5 days after *C. albicans* infection, whereas approximately 70% of mice that received PAF for 3 days after infection survived over 14 days following challenge. This protective effect of PAF was associated with a significant decrease in the number of *C. albicans* in the kidneys ($[7.32 \pm 1.67] \times 10^3$ CFU/kidney for control mice versus $[1.94 \pm 0.48] \times 10^3$ CFU/kidney in the group receiving three daily PAF injections; $P < 0.01$ [Fig. 1B]). Administration of PAF before the infection (up to 5 days) had no protective effect (data not shown). Complete inhibition of PAF-induced resistance was achieved when the PAF antagonist BN50739 (200 μg/mouse) was given intraperitoneally (i.p.) 30 min before each PAF injection (Fig. 2).

Since exogenous PAF increased resistance to *C. albicans* infection, the influence of endogenous PAF on *Candida* resistance was investigated. Administration of BN50739 (200 μg) (single i.p. injection daily) for 3 consecutive days (days 0 to 2) after *C. albicans* infection significantly shortened the survival of the animals from a mean of 5.5 days to 3.4 days ($P < 0.05$) and increased the number of *C. albicans* cells present in the kidneys (from $[5.61 \pm 0.88] \times 10^3$ to $[10.4 \pm 1.8] \times 10^3$ CFU/kidney; $P < 0.05$ [Table 1]). These results suggest that endogenous PAF is released after *C. albicans* infection. Therefore, we determined whether *C. albicans* infection resulted in an increase in the concentration of PAF in the circulation. Mice were infected with 10^7 *C. albicans* cells, and blood was collected at the times indicated. As shown in Fig. 3, *C. albicans* infection resulted in an increase in plasma PAF within 1 h, which reached a peak after 12 h and declined gradually thereafter.

Inhibition of PAF-induced enhanced resistance by anti-TNF-α. Given the facts that PAF and TNF-α influence the synthesis of each other in a positive manner (13, 20, 26, 37, 54) and TNF-α has been shown to be important in protection against systemic candidal infection (34, 51), we investigated whether TNF-α participates in the protective role of PAF in *C. albicans* infection. Anti-TNF-α (2.0 mg) was administered i.p. 10 min before each injection of PAF (1 μg), and control mice received the same concentration of preimmune IgG. As shown in Fig. 4, significant inhibition of the PAF-induced resistance was observed by pretreatment with anti-TNF-α but not with control IgG. The anti-TNF-α pretreatment reduced survival from 60 to 0% (Fig. 4A) and increased the number of *C. albicans* cells in the kidneys from $(0.79 \pm 0.41) \times 10^3$ CFU to $(3.35 \pm 0.71) \times 10^3$ ($P < 0.02$) (Fig. 4B). These results suggest that TNF-α plays an important role in PAF-induced enhanced resistance to *C. albicans* infection. Indeed, administration of anti-TNF-α antibody alone resulted in an enhanced susceptibility to *Candida* infection (Fig. 5). These data further supported a protect effect of TNF-α in candidiasis and confirmed other reports which showed similar results (34, 51).

The previous results and studies by other investigators (20) imply that TNF-α production in vivo is increased or elevated in response to PAF. To clarify this, we determined whether administration of PAF induces the release of TNF-α. Administration of 1 μg of PAF resulted in increased serum TNF-α levels and the expression of TNF-α mRNA in tissue. Maximum production and expression of TNF-α were reached at 1 h (Fig. 6A) and 30 min (Fig. 6B), respectively.

It was next determined if administration of a PAF antagonist could block the increase in serum TNF-α induced by *C. albicans* infection. As shown in Fig. 7, TNF-α was first detected in serum 8 h after infection, and the levels increased progressively

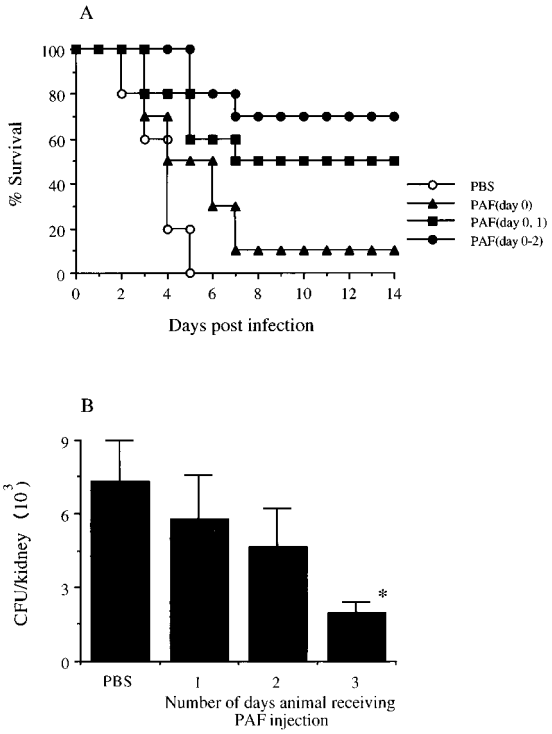

FIG. 1. Effect of PAF administration on survival and the number of *C. albicans* cells in the kidneys in mice infected with *C. albicans*. Mice were infected i.v. with 10^7 ($n = 10$) *C. albicans* cells for survival (A) or with 5×10^6 ($n = 5$) for recovery from kidneys (B) on day 0. Each group of mice was treated i.p. for the indicated number of days with a single injection of PAF (1 μg). *, $P < 0.01$ compared with PBS-treated group.

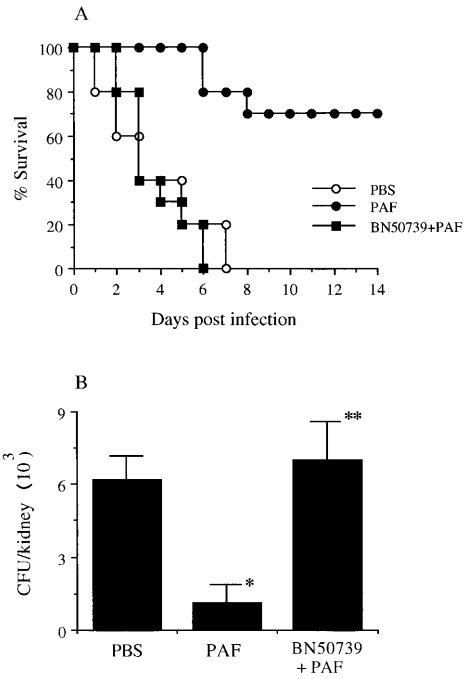

FIG. 2. PAF-induced augmentation of resistance to *C. albicans* injection can be blocked by a PAF antagonist. BN50739 (200 μg) was administered i.p. prior to each injection of PAF (1 μg) for 3 days (days 0 to 2) following *C. albicans* challenge. Mice were infected i.v. with 10^7 ($n = 10$) *C. albicans* cells for survival (A) or with 5×10^6 ($n = 5$) for recovery from kidneys (B) on day 0. Kidneys were removed on day 3, and the number of CFU/kidney was determined. *, $P < 0.01$ compared with PBS-treated group; **, $P < 0.01$ compared with PAF-treated group.

thereafter during the indicated time points. Pretreatment with BN50739 (200 μg) diminished *C. albicans* infection-induced increases in serum TNF-α by more than 40 to 50% ($P < 0.05$), strongly suggesting that the protective effects of PAF against *Candida* are mediated through the actions of TNF-α.

DISCUSSION

This study demonstrates that PAF plays a protective role in host resistance to *C. albicans* infection. The augmentation of resistance to *Candida* was observed when PAF was administered only subsequent to infection. Multiple injections of PAF were found to be more effective than a single injection. In addition, a significantly shortened mean survival time and increased number of *Candida* cells in the kidney were observed when animals were pretreated with a PAF antagonist before infection. The PAF antagonist BN50739 is a synthetic PAF analog; thus, it counteracts the effects of endogenous PAF released in response to *C. albicans* by inhibiting PAF binding to its receptor and the subsequent cellular response. These results support a role for endogenous PAF in the ability of the host to effectively control a candidal infection. The production of endogenous PAF correlates with an enhanced resistance to *C. albicans* infection (Fig. 4).

TABLE 1. Effect of the PAF antagonist BN50739 on the survival and the growth of *C. albicans* in mice infected with *C. albicans*

BN50739[a]	Mean days of survival[b] (range)	CFU/kidney[c] (10^3)
−	5.5 ± 0.57 (4.0–6.2)	5.61 ± 0.88
+	3.4 ± 0.59 (2.8–5.5)[d]	10.36 ± 1.77[d]

[a] Single daily i.p. injections of 200 μg of BN50739 were given for 3 days (day 0 to 2) after infection with *C. albicans* on day 0.
[b] Mice were infected i.v. with 10^7 *C. albicans* cells on day 0.
[c] Mice were infected i.v. with 5×10^6 *C. albicans* cells on day 0. Kidneys were removed on day 3, and the number of CFU was determined.
[d] $P < 0.05$ compared to BN50739 untreated mice.

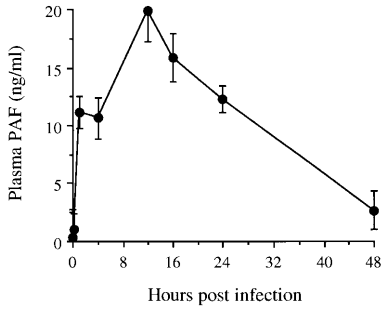

FIG. 3. Plasma PAF levels following *C. albicans* infection. Mice were infected with 10^7 *C. albicans* cells, and blood was collected from each group of mice ($n = 3$ for each point) at the time indicated. Plasma PAF was measured as described in Materials and Methods.

PAF is a potent autacoid mediator produced by a variety of inflammatory cells (12, 15, 36). Although PAF has been demonstrated to be an important mediator of inflammation and endotoxin shock (9), the role of PAF in a protective immune response to a variety of microorganisms, including *C. albicans*, has yet to be determined. In this regard, the present study

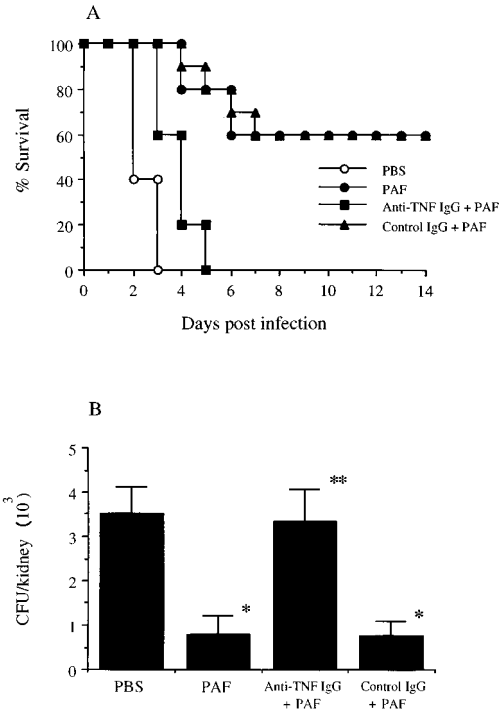

FIG. 4. Abrogation of the PAF-induced enhanced resistance against *C. albicans* infection by anti-TNF-α IgG. Two milligrams of anti-TNF-α IgG or control IgG was given i.p. 10 min prior to each injection of PAF (1 μg) for 3 days (days 0 to 2) after injection of *C. albicans*. In panel A, 10^7 *C. albicans* cells were injected into individual mice ($n = 10$) in the survival experiments; in panel B, 5×10^6 *C. albicans* cells were injected i.p. into mice ($n = 5$) used for recovery experiments. *, $P < 0.01$ compared with PBS-treated group; **, $P < 0.01$ compared with PAF-treated group.

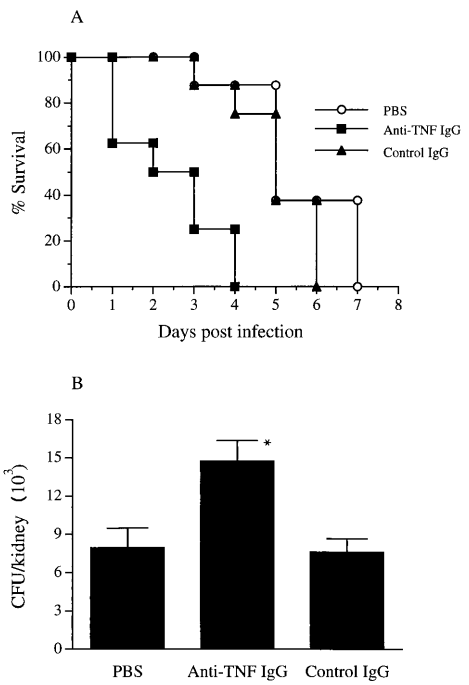

FIG. 5. Enhanced susceptibility to *Candida* infection by administration of anti-TNF-α antibody. Two milligrams of anti-TNF-α IgG or control IgG was given i.p. 10 min prior to injection of 10^7 *C. albicans* cells (A; $n = 8$) or 5×10^6 (B; $n = 5$) *C. albicans* cells. *, $P < 0.01$ compared with control IgG-treated group.

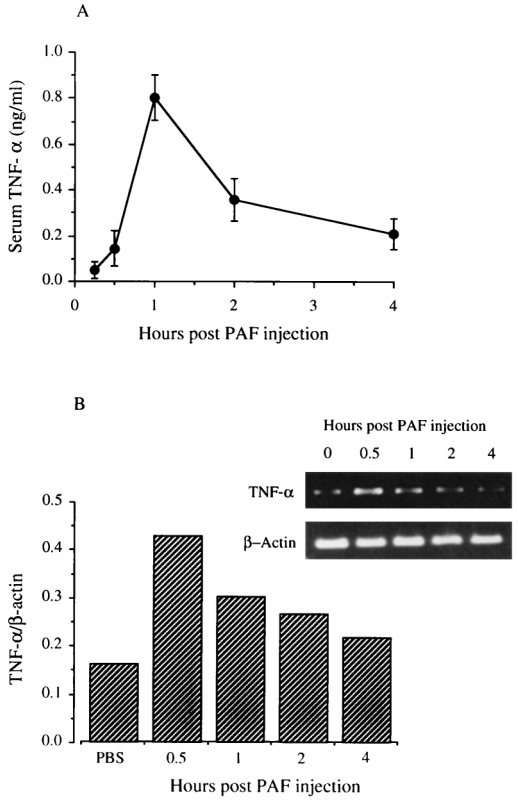

FIG. 6. Kinetics of serum TNF-α (A) and TNF-α mRNA levels in the kidney (B) after injection of PAF. A single injection of PAF (1 μg) was given i.p., and blood and kidneys were collected from each group of mice at the time indicated ($n = 3$ for each point). Serum TNF-α concentrations were determined by ELISA, and kidney TNF-α mRNA expression was analyzed by reverse transcriptase PCR as described in Materials and Methods.

provides the first evidence that PAF has a protective role in systemic candidiasis.

Another important finding of this study is that PAF-mediated protection was profoundly diminished by pretreatment with anti-TNF-α, strongly suggesting that PAF induces the endogenous production of TNF-α following *Candida* infection. Moreover, administration of PAF alone resulted in the elevation of circulating TNF-α levels and an increased expression of tissue TNF-α mRNA (Fig. 6). More importantly, the administration of a potent PAF antagonist to animals diminished *C. albicans*-induced TNF-α production (Fig. 7). However, since the PAF antagonist only partially blocked the production of TNF-α, it is possible that other factors are involved in the PAF-mediated phenomenon. In fact, eicosanoid (20, 56) and IL-1 (11, 26, 54) may influence PAF-mediated TNF-α production. Additional investigations are required to address the question. Collectively, these data strongly suggest that PAF which is produced shortly after *C. albicans* infection leads to the production of TNF-α, which plays a protective role against the infection. We do not know the precise mechanism(s) underlying the interplay between PAF and TNF-α in *Candida* infection. However, given the identification of several cellular components of *C. albicans*, such as mannan (22) and oligomannoside (28), as stimuli for TNF-α production from macrophages, and in this study, the earlier release of PAF than of TNF-α in response to *C. albicans* and the inhibition of *C. albicans* infection-induced TNF-α production by a PAF antagonist, it is possible that the interaction of *C. albicans* and inflammatory cells results in the early release of PAF, leading to subsequent TNF-α production.

PAF and TNF-α stimulate the release of each other via a positive feedback loop in vitro (13, 20, 26, 37, 54). Furthermore, PAF and TNF-α share numerous in vivo biological activities in many pathological conditions, such as lethality in septic shock (7, 14), hematological abnormalities (16, 40), experimental tumor metastases (26), inflammatory bowel diseases (23, 24), and angiogenesis (37). In this respect, our results also support the in vivo (25) as well as in vitro studies demonstrating a positive feedback network between PAF and TNF-α (20).

TNF-α is produced upon challenge with various microorganisms, including bacteria (19, 35, 42), viruses (1, 6), parasites (39, 44), and fungi (50), and plays an important role in host resistance to infection. TNF-α is also produced in vitro (2, 18,

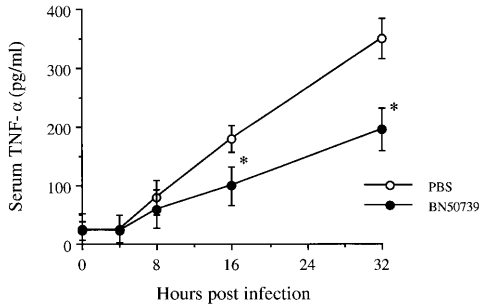

FIG. 7. Effects of BN50739 on serum TNF-α levels at different times after *C. albicans* infection. BN50739 (200 μg) was given i.p. 10 min prior to infection with 10^7 *C. albicans* cells. At the times indicated, blood was collected from each group of mice ($n = 3$ for each point) and analyzed for TNF-α content by ELISA. *, $P < 0.05$ compared with PBS-treated group.

27, 50) as well as in vivo (47, 57) in response to *C. albicans*. It has been shown that TNF-α mediates its beneficial effects in *Candida* infections mainly by activating phagocytic cells (10, 29, 38, 55). At the present time, we do not know whether PAF plays a similar protective role by acting as an inducer of TNF-α in other microbial infections. However, given the observations that TNF-α plays a role in host defense against a variety of experimental microbial infections (50, 39, 52, 53) and that a positive feedback network between PAF and TNF-α exists, it is possible that PAF is an important inducer of substances that are necessary for protective antimicrobial defense responses.

As well as having a role in *Candida* infection, PAF has an important role in the early nonpathogen-specific inflammatory events. Inhibition of endotoxin-induced TNF-α production and death by PAF antagonist (21, 41) suggest that the release of PAF in response to endotoxin precedes that of TNF-α. This notion was further strengthened by our recent observations that PAF is released immediately or shortly after endotoxin injection and induces TNF-α gene expression and production through the activation of multi-nuclear factor κB (26a). Thus, it appears that PAF may be the most proximal mediator regulating the subsequent cascade events in both infection and inflammation.

ACKNOWLEDGMENTS

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