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Tumor Necrosis Factor (TNF) Is Induced in Mice by *Candida albicans*: Role of TNF in Fibrinogen Increase

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One intraperitoneal dose of *Candida albicans* (10^8 CFU) caused a chronic (longer than 2 months), significant elevation of plasma fibrinogen levels (Clauss method) in mice of strain C3H/HeN. Even a small dose (10^6 CFU) resulted in a significant increase in fibrinogen level for 5 days following injection, whereas other blood parameters (leukocytes, erythrocytes, platelets, hematocrit, hemoglobin, blood urea nitrogen, asparate aminotransferase, albumin, alkaline phosphatase, antithrombin III, glucose, calcium, and total protein) measured by standard methods were normal. Blood taken during this period was negative for *C. albicans*. The role of tumor necrosis factor (TNF) in *C. albicans* infections was investigated by measuring the fibrinogen response after the administration of *C. albicans* or recombinant mouse TNF-α. Both challenges resulted in an elevated fibrinogen level. When polyclonal antibodies to mouse TNF-α were given prior to challenge with *C. albicans* or mouse TNF-α, the fibrinogen increase was significantly inhibited. *C. albicans* injections were found to significantly elevate endogenous TNF levels in mice (enzyme-linked immunosorbent assay). It was concluded that *C. albicans* induces TNF in the mouse. Furthermore, these data give evidence which supports a relationship between TNF and the fibrinogen increase induced by *C. albicans*.

We have previously found that a small dose of *Candida albicans*, which had little adverse effect by itself, acted synergistically with *Staphylococcus aureus* to cause shock and death in mice (4). The present study was undertaken to determine how *C. albicans* contributes to this lethal shock synergism. It has been reported that induced tumor necrosis factor (TNF) is responsible for endotoxic shock in the mouse (3). Because *C. albicans* and endotoxin share a number of characteristics (for review, see reference 15), mice infected with *C. albicans* were examined for induced TNF. It is reasonable to suspect that *C. albicans* could induce TNF in vivo because it has been reported recently that *C. albicans* induced TNF production in vitro by human monocytes and natural killer cells (9). TNF has also been shown to potentiate the fungicidal activity of human neutrophils in vitro against *C. albicans* (10, 11). As exogenously administered TNF is known to induce acute-phase proteins such as fibrinogen (12), plasma fibrinogen in infected mice was also measured and the role of TNF in the fibrinogen increase was investigated.

MATERIALS AND METHODS

Mice. Endotoxin-resistant C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and congenic endotoxin-sensitive C3H/HeN mice (18) were obtained from Charles River Laboratories, Wilmington, Mass. Five-week-old male mice weighing 20 to 25 g were used. Animals were caged individually and given food and water ad libitum.

Pathogen. All studies were performed with *C. albicans* L-1 strain from the microbiology laboratory stock culture at Michigan Technological University, Houghton. Cultures used for animal inoculations were grown on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) at 37°C for 48 h, washed, and quantified as described previously (5). *C. albicans* L-1 had an intraperitoneal (i.p.) 50% lethal dose in mice of 3.2×10^8 CFU at the time of this study.

TNF and antibodies to TNF. Recombinant murine TNF-α (rMuTNF-α), rabbit preimmune serum, rabbit polyclonal anti-TNF-α serum (polyclonal anti-rMuTNF-α), and hamster monoclonal anti-rMuTNF-α were purchased from Genzyme Corporation, Boston, Mass. The rMuTNF-α was more than 99% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The specific activity of rMuTNF-α was 4×10^7 U/mg as assayed on L929 cells in the presence of dactinomycin (16). Rabbit polyclonal anti-rMuTNF-α had a neutralizing activity of 10^6 neutralizing units per ml of mouse TNF bioactivity in the L929 assay and was species specific for either native or recombinant murine TNF. Rabbit preimmune serum showed no detectable reactivity with mouse TNF-α (see manufacturer's data sheet). Polyclonal anti-rMuTNF-α showed no detectable reactivity with mouse recombinant interleukin-1 (IL-1), gamma interferon (IFN-γ), or *C. albicans*, either whole cells or cytoplasmic proteins (National Institute for Biological Standards and Controls, London, England) by the immunoblot assay (13). The monoclonal anti-rMuTNF-α, obtained by in vivo immunization of a hamster with purified rMuTNF-α followed by fusion of hamster spleen cells with a hypoxanthine-aminopterin-thymidine-sensitive mouse myeloma cell line (17), had an antibody concentration of 2 mg/ml with 25,000 neutralizing units/ml of mouse TNF bioactivity in the L929 assay. The purified monoclonal antibody used as the capture antibody for the enzyme-linked immunosorbent assay (ELISA) described below showed specific binding to mouse TNF-α and TNF-β and no detectable reactivity with recombinant mouse IFN-α, IFN-β, IFN-γ, or IL-1 (see manufacturer's data sheet).

Animal inoculations. Strict precautions were taken to avoid endotoxin contamination of preparations used for in vivo studies, including the use of nonpyrogenic saline and heating glassware at 160°C for 3 h. *C. albicans*, rMuTNF-α, or refined standard endotoxin from *Salmonella typhimurium* (RIBI ImmunoChem Research Inc., Hamilton, Mont.) was introduced i.p., with each desired dose prepared in 0.2 ml of sterile nonpyrogenic saline (Abbott Laboratories, Chicago, Ill.) and mixed immediately before injection. Polyclonal

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anti-rMuTNF-α (50 μl) was suspended in an equal volume of nonpyrogenic saline and given i.p. 18 h before challenge with *C. albicans* or rMuTNF-α. (Polyclonal anti-rMuTNF-α was found in preliminary studies to be most effective when administered 18 h before challenge with rMuTNF-α [unpublished].) Control animals received an equal volume of nonpyrogenic saline in place of *C. albicans* or rMuTNF-α. To determine whether the polyclonal anti-rMuTNF-α contained any anti-*C. albicans* activity, groups of six mice were treated with the polyclonal anti-rMuTNF-α or nonpyrogenic saline 18 h prior to injection with *C. albicans*. Animals were sacrificed at 24 h after infection, and CFU were determined in the homogenized, suspended liver and pancreas from each animal as described previously (5).

Clinical pathology. Temporal changes in plasma fibrinogen levels were determined from blood specimens obtained by tail clip before inoculation and at selected times after inoculation. Samples (0.1 ml) were collected in capillary tubes (Clay Adams, Parsippany, N.J.) containing 1/10 volume of 0.13-mol/liter sodium citrate. All specimens were centrifuged, and plasma fibrinogen was measured by the thrombin clotting time method of Clauss (7). Reagents for fibrinogen determination were obtained in kit form (American Bioproducts, Parsippany, N.J.). To obtain blood chemistry and hematology values for animals treated i.p. with 10^6 CFU of *C. albicans* and control animals, blood was obtained 24 h postinoculation by cardiac puncture from mice anesthetized by CO_2; 0.5 ml was transferred to a tube (serum separator 5960, Becton Dickinson [BD], Rutherford, N.J.) for blood chemistry analysis, 0.2 ml was transferred to a whole-blood collector with EDTA (BD 5961) for hematology, and 0.5 ml was transferred to a tube (BD 5960) with 1/10 volume of 0.13-mol/liter sodium citrate for fibrinogen and antithrombin III (ATIII) (chromogenic method [1]). Reagents for ATIII (American Bioproducts) were available in kit form. In addition, 0.1 ml of blood was added to 0.9 ml of sterile saline, spread on the surface of a Sabouraud dextrose agar plate, and incubated at 37°C for 48 h, after which the plate was examined for *C. albicans* colonies (two such determinations were done per mouse). Blood was analyzed chemically, and hematology was done by Calumet Public Hospital (Calumet, Mich.) under the direction of P. Grill.

TNF assay. TNF was quantitated by a sandwich-type ELISA as described previously (17). Briefly, microtiter plate wells were precoated with 200 ng of hamster monoclonal anti-rMuTNF-α in 0.1 M carbonate buffer (pH 9.6). Unoccupied binding sites in the wells were blocked as described before (14) with 0.1 M carbonate buffer containing 0.1% bovine serum albumin and 2% goat serum (Sigma Chemical Co., St. Louis, Mo.). Wells were incubated with 100 μl of a TNF source (serum) in triplicate for 1 h at 23°C and then exposed to rabbit polyclonal anti-rMuTNF-α diluted 1/200 in phosphate-buffered saline containing 0.1% bovine serum albumin and 2% goat serum. Plates were developed by using peroxidase-labeled goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (Sigma Chemical Co.). Selected amounts of rMuTNF-α suspended in 100 μl of mouse serum were used to construct standard curves to accompany each experiment. The assay was determined to significantly detect TNF concentrations as low as 30 pg/ml. When tested in the ELISA, no detectable reactivity was found with *C. albicans*, either whole cells or cytoplasmic proteins, recombinant mouse IL-1, or recombinant mouse IFN-γ (Genzyme Corp.). An ELISA detecting mouse TNF-α and mouse TNF-β (Genzyme product 1509-00) highly similar to ours, employing the same capture antibody and a rabbit polyclonal antibody made against the same rMuTNF-α, showed no detectable reactivity when tested with recombinant mouse IL-1, IL-2, IL-3, IL-4, IL-5, or granulocyte-monocyte colony-stimulating factor (see manufacturer's data sheet). Since this ELISA is nearly identical to ours, it is likely that ours too detects mouse TNF-β as well as mouse TNF-α.

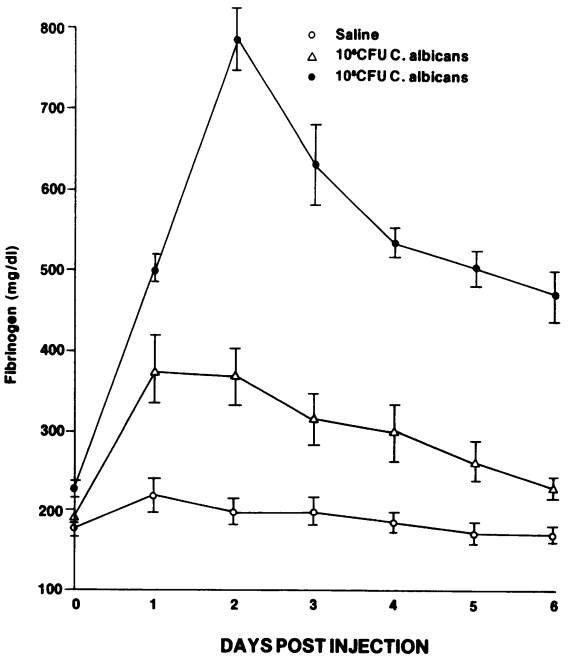

FIG. 1. Fibrinogen levels in mice given 10^6 or 10^8 CFU of *C. albicans* or saline i.p. (six mice per group). Error bars indicate ±1 SE.

Blood was obtained from mice by cardiac puncture at selected times postinoculation, with serum removed after centrifugation. When peak levels of TNF induced by endotoxin and *C. albicans* were compared, serum was sampled as follows. Serum from mice treated with endotoxin was obtained 1.5 h after inoculation. It has been shown by others (20) and confirmed in our laboratory that endotoxin-induced serum levels of TNF peak 1 to 2 h after inoculation and return to normal by 6 h after inoculation. Maximum levels of *C. albicans*-induced serum TNF were found at 24 h after infection (see Results section).

Statistical analysis. Data were analyzed by the Student unpaired *t* test or analysis of variance (Dunnett's test). For the data which did not meet the assumption of homoscedasticity, nonparametric tests were used (Mann-Whitney test, Kruskal-Wallis test, and Dunn's procedure) (19). All experiments were carried out more than once with reproducible results.

RESULTS

Fibrinogen increase following *C. albicans* infection. One i.p. dose of *C. albicans* (10^8 CFU) caused a chronic (at least 2 months) elevation in plasma fibrinogen in C3H/HeN mice. Even a small dose of *C. albicans* (10^6 CFU) increased fibrinogen levels for 5 days postinjection (Fig. 1). Although fibrinogen levels returned to normal 6 days after injection with 10^6 CFU of *C. albicans*, fibrinogen in mice receiving the

TABLE 1. Fibrinogen increase in C3H/HeN and C3H/HeJ mice 48 h after i.p. injection of 10^6 CFU of *C. albicans*

Mouse strain	Treatment	Mean fibrinogen level[a] (% change after 48 h) ± SE
C3H/HeJ	*C. albicans*	+35.8 ± 8.0[b]
	Saline	−12.5 ± 8.4
C3H/HeN	*C. albicans*	+32.6 ± 6.4[b]
	Saline	−12.2 ± 3.4

[a] Fibrinogen was measured at time of treatment and 48 h after treatment.
[b] Significantly elevated compared with saline control ($P < 0.0025$ by the Student unpaired t test).

10^8-CFU dose was still elevated (493 mg/dl) 2 months after injection (not shown). In a separate experiment, it was found that endotoxin-resistant mice, C3H/HeJ, responded to 10^6 CFU of *C. albicans* with a significant fibrinogen increase in a manner similar to the C3H/HeN mouse strain (Table 1).

Among the animals inoculated with 10^6 CFU of *C. albicans*, blood drawn from six C3H/HeN mice was examined for fibrinogen, ATIII, leukocytes, erythrocytes, platelets, hematocrit, hemoglobin, blood urea nitrogen, asparate aminotransferase, albumin, alkaline phosphatase, glucose, calcium, and total protein 24 h after injection. Except for fibrinogen, all parameters tested were normal and did not differ from those of saline-treated control animals. Blood from each animal (0.2 ml) was cultured 24 h after injection and found to be negative for *C. albicans*. Animals receiving 10^6 CFU of *C. albicans* gave no visible sign of disease.

TNF elevation following *C. albicans* injection. C3H/HeN mice (six to eight per group) were inoculated i.p. with 10^6, 10^7, or 10^8 CFU of *C. albicans* or saline, and serum concentrations of TNF were measured with the ELISA 2.5 h after infection. Concentrations of TNF were 119 ± 13, 190 ± 48, and 308 ± 98 pg/ml in mice injected with 10^6, 10^7, and 10^8 CFU of *C. albicans*, respectively (data represent the mean value ± standard error). These values were significantly elevated compared with the saline control group, which had a TNF concentration of 13 ± 13 pg/ml ($P < 0.001$ by Kruskal-Wallis test).

In another experiment, groups of C3H/HeN and C3H/HeJ mice were inoculated i.p. with two doses of *C. albicans* (10^6 or 10^8 CFU) or endotoxin (1 or 10 μg), and serum levels of TNF were measured at the time of peak induction. Serum from both endotoxin-sensitive and -resistant mice inoculated with *C. albicans* was equally positive for TNF, whereas endotoxin induced TNF only in the endotoxin-sensitive strain (Table 2). It was also noted that the C3H/HeN animals inoculated with *C. albicans* had a considerably higher serum concentration of TNF at 24 h than at 2.5 h postinfection (see above). It was determined separately that the peak levels of TNF found 24 h after infection diminished by 31 ± 19% by 48 h after infection.

Fibrinogen response following treatment of mice with polyclonal rabbit anti-rMuTNF-α. The relationship between TNF and the fibrinogen increase induced by *C. albicans* was investigated in C3H/HeN mice by measuring the fibrinogen response after administration of 10^6 CFU of *C. albicans* with and without polyclonal anti-rMuTNF-α. When polyclonal anti-rMuTNF-α was given 18 h prior to challenge with *C. albicans*, animals were fully protected from the fibrinogen increase at 24 h postinfection and partially protected for the following 5-day testing period (Fig. 2). In a separate experiment, groups of six mice received preimmune serum or saline 18 h prior to *C. albicans* infection. Preimmune serum offered no protection from the fibrinogen increase induced by *C. albicans* (fibrinogen increase of 34 ± 7.7% and 33 ± 21% at 24 h and 50 ± 19% and 60 ± 19% at 48 h for the saline and preimmune serum treatment groups, respectively).

TABLE 2. Peak TNF levels in serum in C3H/HeN and C3H/HeJ mice after i.p. injection of *C. albicans* or endotoxin[a]

Treatment	Mouse strain	Mean TNF concn (pg/ml) ± SE
C. albicans		
10^6 CFU	C3H/HeN	560 ± 238
	C3H/NeJ	468 ± 194
10^8 CFU	C3H/HeN	1,717 ± 174
	C3H/HeJ	1,711 ± 187
Endotoxin		
1 μg	CeH/HeN	7,738 ± 107[b]
	C3H/HeJ	10 ± 10
10 μg	C3H/HeN	17,400 ± 3,482[b]
	C3H/HeJ	206 ± 62
Saline	C3H/HeN	28 ± 7[c]
	C3H/HeJ	16 ± 5[c]

[a] Serum was obtained at times of peak induction of TNF, which was 1.5 h for endotoxin-induced TNF and 24 h for *C. albicans*-induced TNF (see text). There were six to eight mice per group.
[b] Significantly different from C3H/HeJ group; $P < 0.02$ (Mann-Whitney test).
[c] Saline control groups were included with each experimental group. This value represents the mean of the combined saline control groups.

The following experiment was done to determine whether polyclonal anti-rMuTNF-α possessed activity against *C. albicans* or increased its clearance. The *C. albicans* CFU titer was determined in the livers and pancreas from antibody-treated and saline-treated mice 24 h after i.p. infection with 10^6 CFU of *C. albicans*. No significant differences were found between the six animals in the antibody-treated group (liver, 3.7 (±1.4) × 10^3 CFU; pancreas, 1.2 (±0.3) × 10^4

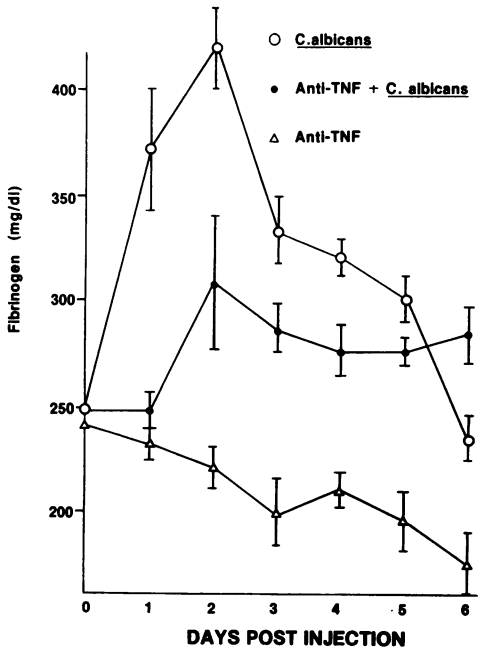

FIG. 2. Fibrinogen levels in mice treated with anti-rMuTNF-α 18 h prior to inoculation with 10^6 CFU of *C. albicans* (●), saline 18 h prior to inoculation with *C. albicans* (○), or anti-rMuTNF-α 18 h prior to inoculation with saline (△) (six mice per group). Error bars indicate ±1 SE.

TABLE 3. Effect of rMuTNF-α on plasma fibrinogen in C3H/HeN mice with and without prior treatment with polyclonal anti-rMuTNF-α

Treatment group (n = 6)	Pretreatment	Mean plasma fibrinogen level[a] (% change after 24 h) ± SE
rMuTNF-α	Preimmune serum	+45.3 ± 10.6
	Saline	+45.6 ± 4.8
	Polyclonal anti-rMuTNF-α	+23.9 ± 4.7[b]
Saline	Polyclonal anti-rMuTNF-α	−2.6 ± 4[b]
	Saline	+2.7 ± 4.6

[a] Fibrinogen was measured at time of treatment and 24 h after treatment (saline or 1 μg of TNF).
[b] Significantly different ($P < 0.0025$) from rMuTNF-α–preimmune serum group by analysis of variance (Dunnett's test).

CFU) and the six animals in the saline control group (liver, 3.2 (±1.5) × 10^3 CFU; pancreas, 7.2 (±3.0) × 10^3 CFU).

In another experiment, plasma fibrinogen examined 24 h after an i.p. infection of 1 μg of rMuTNF-α in C3H/HeN mice was found to increase, whereas pretreatment with polyclonal anti-rMuTNF-α 18 h before rMuTNF-α exposure significantly reduced this fibrinogen increase (Table 3).

DISCUSSION

One relatively large but nonlethal i.p. dose (10^8 CFU) of *C. albicans* caused a chronic elevation of plasma fibrinogen in C3H/HeN mice for at least 2 months. A smaller dose of *C. albicans*, 10^6 CFU, caused a transient (5-day) increase in fibrinogen levels. When groups of mice were injected with 10^8, 10^7, or 10^6 CFU of *C. albicans* and levels of TNF were determined, it was found that the serum concentrations of TNF in all of the infected groups were elevated by 2.5 h after infection. When infected animals (10^6 and 10^8 CFU) were sampled 24 h postinfection, the TNF concentration in serum was four times that observed at 2.5 h postinfection.

Since, in addition to *C. albicans*, an injection of rMuTNF-α i.p. also caused an increase in fibrinogen, and since polyclonal anti-rMuTNF serum protected animals injected with either *C. albicans* or rMuTNF-α from the fibrinogen increase, the possibility that *C. albicans* induced endogenous TNF which in turn caused the fibrinogen increase is reasonable. The finding that TNF levels in mice treated with *C. albicans* were elevated further supports a relationship between TNF induced by *C. albicans* and the fibrinogen increase. Since TNF induction by *C. albicans* was also observed in C3H/HeJ endotoxin-resistant mice, it is unlikely that endotoxin played a role in this response.

Since endotoxin does not induce TNF in the C3H/HeJ strain (2), our finding that *C. albicans* did induce TNF in the endotoxin-resistant strain suggests that the induction of TNF by *C. albicans* is under a different mechanism of control than the induction of TNF by endotoxin. This possibility is further supported by the time of appearance of TNF after exposure. Whereas endotoxin induces a short pulse of TNF peaking 1.5 h after infection (20), TNF induced by *C. albicans* is undetectable at 1.5 h (our unpublished data) and continues to increase for at least 24 h after injection. Comparative studies of TNF induction by endotoxin and *C. albicans* are currently in progress in this laboratory.

It is interesting that the mice receiving the small nonlethal dose (10^6 CFU) of *C. albicans*, in spite of their significant serum concentration of TNF, appeared to be well, with no change in any clinical parameter tested other than fibrinogen. It is possible that mice receiving the small dose of *C. albicans* remained healthy because the TNF concentration in serum of 560 pg/ml was too low to cause pathological changes. In a report on mice experiencing TNF-dependent endotoxic shock, mice received a lethal dose of 400 μg of endotoxin (3); studies quantitating TNF revealed that mice receiving 400 μg of endotoxin developed TNF levels in serum of nearly 20 ng/ml (20). Our studies suggest that the fibrinogen level is relatively sensitive to low levels of TNF.

The focus of this study was to determine whether TNF was induced in mice by *C. albicans* and whether TNF was related to the fibrinogen increase induced by *C. albicans*. As polyclonal anti-rMuTNF-α totally protected animals from the fibrinogen response 24 h postinfection, it is reasonable to assume that TNF is important early in this response. However, the inability of anti-rMuTNF to fully abrogate the fibrinogen response on days 2 through 5 after inoculation of 10^6 CFU of *C. albicans* suggests that additional mechanisms may be operative at these later times. TNF, when injected into rabbits, induces a fever in minutes and several hours later induces IL-1 along with a second, IL-1-induced fever (8). In addition to pyrogenicity, TNF and IL-1 share the ability to induce acute-phase proteins such as fibrinogen (12). IL-6 has also been reported to influence acute-phase protein production (6). It is possible that the mechanism responsible for the peak fibrinogen increase observed 48 h postinfection, which is only partially reduced by polyclonal anti-TNF, is a result of additional cytokines such as IL-1 and IL-6. The appearance of TNF, IL-1, and IL-6 with time, currently under study in our laboratory, will be important in determining the more complex mechanism operative at 2 to 5 days postinfection.

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