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Tumor Necrosis Factor and Interleukin-6 in *Candida albicans* Infection in Normal and Granulocytopenic Mice

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We administered a neutralizing monoclonal antibody to tumor necrosis factor (TNF) during infection with *Candida albicans* in normal and granulocytopenic mice. Mice were rendered granulocytopenic ($<0.1 \times 10^9$ granulocytes per liter) with cyclophosphamide. Growth of *C. albicans* from the kidneys was significantly increased in normal mice treated with the antibody to TNF, compared with that in control mice, after 36 h ($3.6 \times 10^4 \pm 1.2 \times 10^4$ CFU per kidney versus $9.1 \times 10^3 \pm 6.2 \times 10^3$ CFU per kidney; $P < 0.05$) and after 72 h ($3.7 \times 10^6 \pm 2.7 \times 10^6$ CFU per kidney versus $2.3 \times 10^4 \pm 1.3 \times 10^4$ CFU per kidney; $P < 0.01$). In granulocytopenic mice, the antibody to TNF had no effect on the growth of *C. albicans* from the kidneys. Furthermore, our study showed that the cytokines TNF and interleukin-6 (IL-6) were produced in a dose-dependent manner during *C. albicans* infection. TNF was detectable between 6 and 60 h, with peak levels at 24 h. Both TNF and IL-6 levels were significantly higher in cyclophosphamide-treated mice than in normal mice. Heat-inactivated *C. albicans* induced a TNF response different from that induced by viable *C. albicans*, with an early peak occurring at 3 to 4 h and declining to nondetectable levels after 15 to 24 h. Peak levels of TNF obtained with heat-inactivated *C. albicans* were lower than those obtained with viable *C. albicans*. Our study demonstrates that TNF and IL-6 are produced systemically during *C. albicans* infection and suggests that TNF is essential for granulocyte antifungal activity in vivo.

Tumor necrosis factor (TNF) is a multifunctional cytokine with a dual pathogenetic role in infections. High levels of TNF have been correlated with a fatal outcome in human meningococcal septicemia (37), and there is firm experimental evidence that TNF is a mediator in septic shock (30, 31). TNF has also been shown to mediate detrimental effects in infections in the absence of granulocytes, as the administration of an antibody to TNF conferred protection against a lethal *Pseudomonas aeruginosa* infection in granulocytopenic rats (22). On the other hand, beneficial effects of TNF have been demonstrated in listeriosis. A sublethal infection with *Listeria monocytogenes* in normal mice developed into a lethal one after the administration of an antibody to TNF, and the growth of *L. monocytogenes* from the livers and spleens of infected, antibody-treated animals was increased (11, 20).

In infections with *Candida albicans*, the cytokine interleukin-1 (IL-1) has been reported to have a protective effect in both normal (19) and granulocytopenic (17, 23, 33) mice. TNF and gamma interferon were shown to potentiate the growth-inhibiting activity of human neutrophils against *C. albicans* in vitro (5), and TNF increased neutrophil fungicidal activity, the production of oxygen radicals, and the release of lysosomal enzymes (9). However, a recent study reported that TNF reduced in vitro neutrophil fungicidal activity against *C. albicans* hyphae, even though the early respiratory burst responses were significantly enhanced (4). Furthermore, *C. albicans* has been reported to induce TNF production in mice (24, 34) and in vitro by human monocytes and natural killer cells (6).

The granulocytopenic host is particularly susceptible to infections with *C. albicans* (2, 14, 16), a fact that indicates a crucial in vivo role for neutrophils in these infections. In this study, we developed a mouse model to examine the production of the cytokines TNF and IL-6 and the interaction between TNF and granulocytes in infections with *C. albicans*. The results demonstrate that TNF and IL-6 are systemically produced during *C. albicans* infections. Neutralization of TNF with an anti-TNF antibody resulted in an increased proliferation of *C. albicans*, and this effect was not present in cyclophosphamide-induced granulocytopenia.

MATERIALS AND METHODS

Mice. Female NMRI mice weighing 22 to 24 g (Bomholt Gård, Ry, Denmark) were caged in groups of 10 and allowed to eat and drink ad libitum.

Granulocytopenia. Mice were rendered granulocytopenic ($<0.1 \times 10^9$ granulocytes per liter) by intraperitoneal (i.p.) injections of cyclophosphamide (Hydro Pharma, Oslo, Norway) (200 mg/kg) 84 and 12 h before inoculation of the fungi. Granulocytopenia occurred at the time of the second injection of cyclohosphamide and lasted for 6 to 7 days (data not shown). Leukocytes were counted in a Coulter Counter (model ZF; Coulter Electronics Ltd., Luton, United Kingdom), and differential counts were obtained with peripheral blood smears stained with May-Grünewald-Giemsa stain. May-Grünewald-Giemsa-stained bone marrow smears from the femurs were examined to confirm the suppression of granulocytopoiesis.

C. albicans. Pathogenic *C. albicans* was originally isolated in cultures of blood from a patient suffering from fungal septicemia. The isolate was identified as *C. albicans* on the basis of common laboratory criteria, including a positive germ tube test and, in addition, by the use of ATB 32C (API System, la Balme les Grottes, Montalieu, France). The isolate was cultured on Sabouraud dextrose agar plates (Oxoid Unipath Ltd., Basingstoke, United Kingdom) for 24 h at 37°C, harvested, washed once in pyrogen-free saline, resuspended, and stored at −70°C in aliquots at a concentration of 10^9 CFU of *C. albicans* per ml. Viable counts were

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determined by inoculating duplicate serial dilutions onto Sabouraud dextrose agar plates and counting the CFU after 48 h of incubation at 37°C. The injected dose of *C. albicans* refers to the number of colonies determined before freezing. Viability after thawing was reduced to 60 to 65%. Heat-inactivated *C. albicans* was prepared by heating to 56°C for 30 min, a treatment that reduced viability to 0%. The fungi were injected i.p. in 0.5 ml of pyrogen-free saline. At the time of injection, a sample was inoculated onto blood and chocolate agar plates and incubated at 37°C for 48 h to detect bacterial contamination. In preliminary experiments, doses of 5×10^8 and 1×10^8 CFU of *C. albicans* caused the death of 50% of normal and granulocytopenic mice ($n = 10$), respectively, between days 2 and 6 after infection.

Growth of *C. albicans*. The growth of *C. albicans* from the kidneys was determined 36 and 72 h after the mice were infected. The right kidney was aseptically removed immediately after the mice were killed, weighed, homogenized in pyrogen-free saline, and grown in duplicate serial dilutions on Sabouraud dextrose agar plates at 37°C for 48 h. In preliminary experiments including 20 mice at both 36 and 72 h, we found no difference in the numbers of *C. albicans* between the right and left kidneys. Identical results were obtained whether the growth of *C. albicans* was calculated as CFU per kidney or CFU per gram of kidney.

Blood sampling. Mice were anesthetized with a 1:1 mixture of midazolam (Dormicum; F. Hoffmann-La Roche & Co. AG, Basel, Switzerland) and fentanyl-fluanison (Hypnorm; Janssen Pharmaceutica, Beerse, Belgium) and bled by cardiac puncture. Blood samples were collected in sterile tubes and allowed to clot on ice before centrifugation. Individual serum samples were then frozen at −20°C until they were assayed for TNF and IL-6 activities.

Antibodies. A hamster-derived anti-murine TNF monoclonal antibody (MAb) (designated TN3 19.12) that neutralizes natural murine TNF (28) was kindly provided by Celltech (Slough, United Kingdom). The antibody concentration was 5.05 mg/ml, and the endotoxin concentration was <0.5 endotoxin units per ml, as determined by a *Limulus* lysate gelation assay. The antibody was given i.p. in a dose of 20 mg/kg 1 h prior to the injection of *C. albicans*. Preliminary experiments demonstrated that serum TNF was completely neutralized by this dose of antibody in both normal and granulocytopenic mice during the first 72 h of infection with *C. albicans*. Furthermore, sera from mice treated with TN3 19.12 were able to neutralize levels of TNF higher than the peak levels observed during a *C. albicans* infection until 9 to 10 days after the administration of TN3 19.12. The half-life of the antibody in mice after i.p. administration has been determined to be 7 days (28).

A hamster-derived MAb (L2 3D9; Celltech) directed against murine recombinant IL-2 does not react with natural murine IL-2 and was given i.p. to control animals at a dose of 20 mg/kg. In our first series of experiments, including the observation of lethality and the determination of growth from the kidneys at 36 and 72 h in normal and granulocytopenic mice, no difference was observed between controls treated with an irrelevant antibody or saline. Saline was subsequently injected into control animals.

TNF and IL-6 bioassays. Serum TNF levels were determined by the WEHI 164 clone 13 cytotoxicity bioassay, which is capable of detecting 0.2 pg of murine TNF per ml of serum (8). The cells were incubated in the presence of serially diluted serum samples, and cytotoxicity was measured after 24 h of incubation with an MTT colorimetric assay (18). The concentration of TNF in serum samples was calculated on the basis of cellular death in the presence of a murine recombinant TNF standard (provided by Genentech Inc., South San Francisco, Calif.). The cytotoxicity induced by serum samples was completely abolished in the presence of TN3 19.12, a result that demonstrated the specificity of the observed activity.

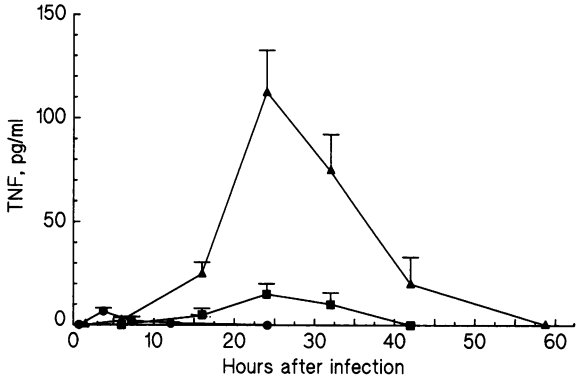


FIG. 1. Kinetics of TNF production. A dose of 3×10^8 CFU of viable *C. albicans* was given to normal mice (■), a dose of 5×10^7 CFU of viable *C. albicans* was given to granulocytopenic mice (▲), and a dose of 5×10^8 CFU of heat-inactivated *C. albicans* was given to granulocytopenic mice (●). Doses of 5×10^7 and 1×10^6 CFU of viable *C. albicans* did not induce a TNF response in normal and granulocytopenic mice, respectively. At the indicated times, six mice were sacrificed. Serum samples from individual mice were tested in triplicate for TNF activity as outlined in Materials and Methods. Data are presented as means ± SEM ($n = 6$). TNF levels were significantly higher in granulocytopenic mice than in normal mice at 16 h ($P < 0.01$), at 24 h ($P < 0.01$), and at 32 h ($P < 0.01$). One of three separate experiments with the same results is shown.

Serum IL-6 levels were determined with murine hybridoma cell line B13.29 clone B9, which is dependent on IL-6 for growth (1). Cell growth was measured after 72 h of incubation with the MTT colorometric assay. The concentration of IL-6 in serum samples was calculated on the basis of cellular growth in the presence of a human recombinant IL-6 standard (gift from L. A. Aarden, University of Amsterdam, Amsterdam, The Netherlands). The cellular growth induced by serum samples was completely abolished in the presence of a rat-derived anti-murine IL-6 MAb designated 6B4 (gift from J. van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium).

Statistical analysis. The significance of differences between numbers of *C. albicans* and cytokine levels in experimental groups and control groups was determined with the Mann-Whitney U test. *P* values were based on two-sided tests. Data are expressed as means ± standard errors of the means (SEM).

RESULTS

Kinetics of TNF and IL-6 production. After a dose of 5×10^7 CFU of *C. albicans* given i.p. to granulocytopenic mice, TNF was detected in serum after 6 h, peaked at 24 h, and gradually declined to nondetectable levels after 60 h (Fig. 1). After the first 72 h, TNF production was determined daily until day 15. No second peak of TNF production was observed. The kinetics of TNF production in the serum of normal mice showed a similar pattern; however, the levels in serum were significantly lower, even though a higher dose (3×10^8 CFU) of *C. albicans* was injected. Doses of 1×10^6

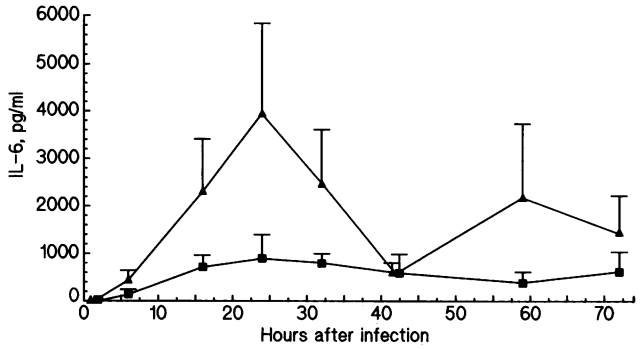


FIG. 2. Kinetics of IL-6 production. A dose of 3×10^8 CFU of *C. albicans* was given to normal mice (■), and a dose of 5×10^7 CFU of *C. albicans* was given to granulocytopenic mice (▲). Procedures were as described in the legend to Fig. 1. Data are presented as means ± SEM ($n = 6$). IL-6 levels were significantly higher in granulocytopenic mice than in normal mice at 24 h ($P < 0.05$). One of three separate experiments with the same results is shown.

and 5×10^7 CFU of *C. albicans* did not induce a TNF response in granulocytopenic and normal mice, respectively.

The pattern of IL-6 production showed considerable variation, as evident from the standard errors in Fig. 2. IL-6 production was observed from 6 h to 3 days, and the highest levels in serum occurred after 24 h. Significantly higher levels were detected in cyclophosphamide-treated mice than in normal mice at 24 h.

TNF and IL-6 were not detected in sera from uninfected mice, either normal or cyclophosphamide treated ($n = 10$).

Heat-inactivated *C. albicans*. A dose of 5×10^8 CFU of *C. albicans* was heat inactivated and administered to cyclophosphamide-treated mice. The kinetics of TNF production were different from those induced by viable *C. albicans*, as peak levels, which were very low, occurred in serum at 3 to 4 h and rapidly declined (Fig. 1). Furthermore, there was no detectable TNF response as early as 90 min after the inoculation of heat-inactivated *C. albicans*, a result that distinguishes these kinetics from those of endotoxin stimulation.

Production of TNF and IL-6 in normal and granulocytopenic mice. An explanation of the higher production of TNF in granulocytopenic mice might be extensive in vivo proliferation of *C. albicans* in the abscence of granulocytes and thus a higher effective dose. To examine this possibility, we injected a 50% lethal dose (LD_{50}) of *C. albicans* into normal and granulocytopenic mice (5×10^8 and 1×10^8 CFU of *C. albicans*, respectively). The TNF response was determined at 24 h. We still observed significantly higher levels of TNF in granulocytopenic mice than in normal mice (156.4 ± 29.7 pg/ml versus 5.2 ± 1.3 pg/ml; $P < 0.01$). Similarly, we found higher serum IL-6 levels at 24 h in granulocytopenic mice than in normal mice after the injection of an LD_{50} of *C. albicans* (5,378.1 ± 1,227.3 pg/ml versus 1,056.3 ± 326 pg/ml; $P < 0.05$).

Effect of TNF on granulocyte activity against *C. albicans*. The effect of TNF on the in vivo growth of *C. albicans* was evaluated by determining the number of *C. albicans* grown from the kidneys after treatment with an MAb to TNF. Preliminary experiments showed that viable counts of *C. albicans* were 10- to 100-fold lower in the liver and spleen than in the kidneys, consistent with the reports of others (25). Furthermore, the administration of TN3 19.12 did not cause any redistribution of organisms among the above-

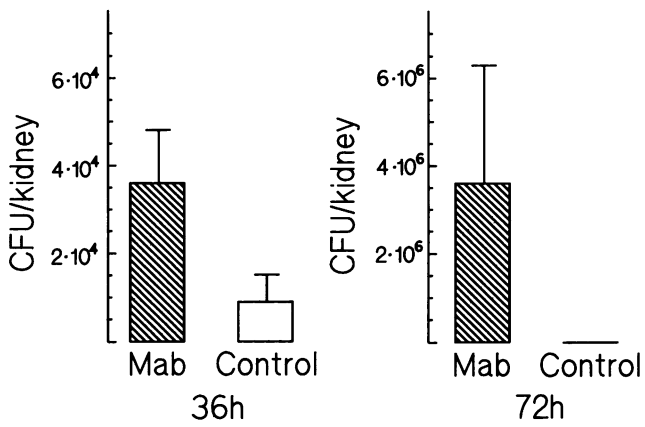


FIG. 3. Effect of an MAb to TNF on the growth of *C. albicans* from the kidneys of normal mice after the administration of a dose of 10^8 CFU of *C. albicans*. The levels of growth of *C. albicans* were significantly higher in antibody-treated mice than in control mice at 36 h ($3.6 \times 10^4 \pm 1.2 \times 10^4$ CFU per kidney versus $9.1 \times 10^3 \pm 6.2 \times 10^3$ CFU per kidney; $P < 0.05$) and at 72 h ($3.7 \times 10^6 \pm 2.7 \times 10^6$ CFU per kidney versus $2.3 \times 10^4 \pm 1.3 \times 10^4$ CFU per kidney; $P < 0.01$). Data are presented as the mean number of colonies cultured from 10 mice ± the SEM. One of four separate experiments with the same results is shown.

mentioned target organs (data not shown). Normal mice were infected with 10^8 CFU of *C. albicans*. The number of *C. albicans* grown from the kidneys in normal mice was significantly higher in the group treated with the MAb to TNF than in the control group at both 36 h ($3.6 \times 10^4 \pm 1.2 \times 10^4$ CFU per kidney versus $9.1 \times 10^3 \pm 6.2 \times 10^3$ CFU per kidney; $P < 0.05$) and 72 h ($3.7 \times 10^6 \pm 2.7 \times 10^6$ CFU per kidney versus $2.3 \times 10^4 \pm 1.3 \times 10^4$ CFU per kidney; $P < 0.01$) after infection (Fig. 3). To investigate the role of the granulocytes in this TNF-dependent antifungal activity, we infected granulocytopenic mice with 5×10^7 CFU of *C. albicans* and determined the growth of the organisms from the kidneys. In these experiments, we found no difference in

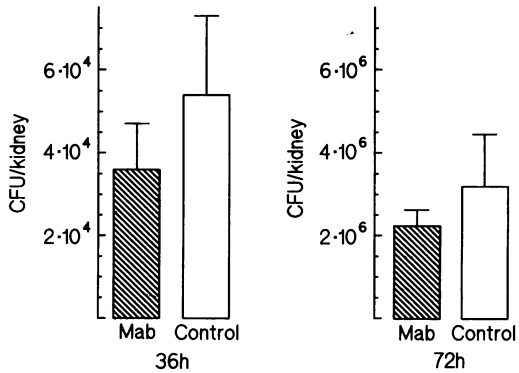
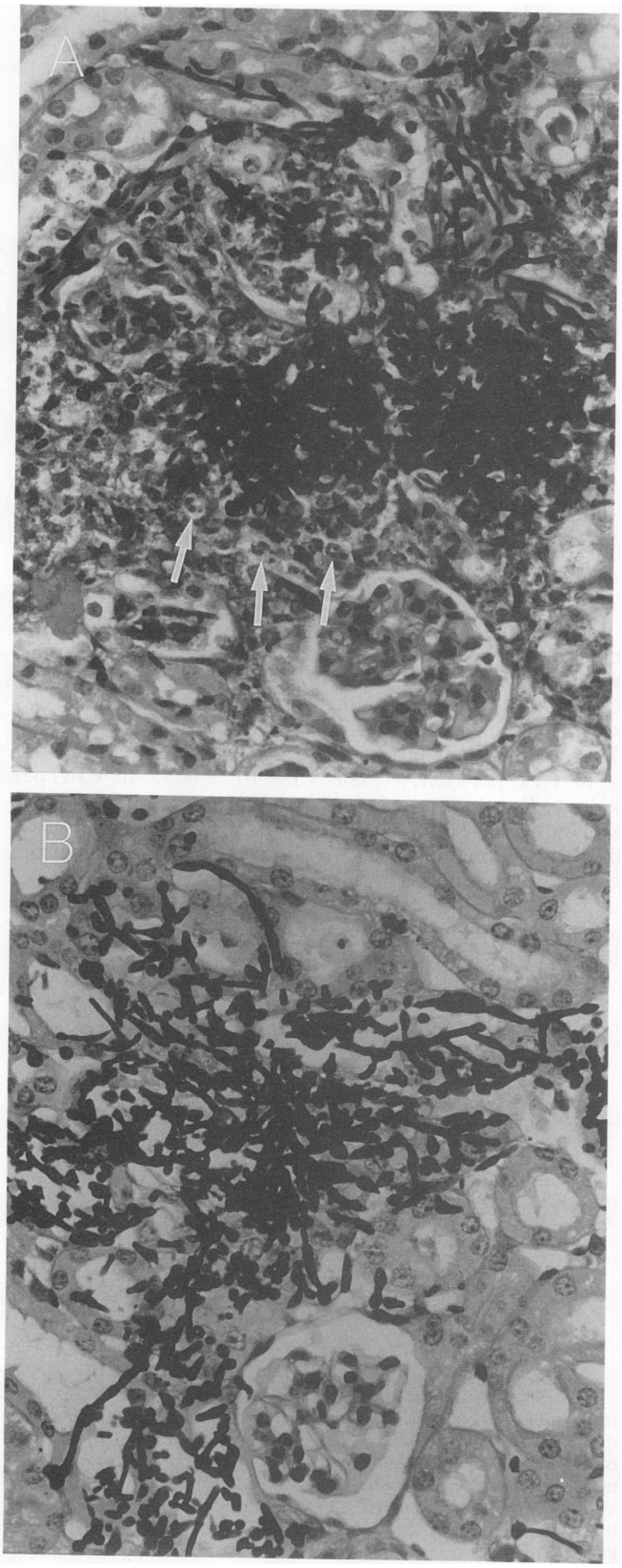


FIG. 4. Effect of an MAb to TNF on the growth of *C. albicans* from the kidneys of granulocytopenic mice after the administration of a dose of 5×10^7 CFU of *C. albicans*. The difference in the growth of *C. albicans* between antibody-treated mice and control mice was not significant at either 36 h ($3.7 \times 10^4 \pm 1.1 \times 10^4$ CFU per kidney versus $5.3 \times 10^4 \pm 1.9 \times 10^4$ CFU per kidney) or 72 h ($2.3 \times 10^6 \pm 0.3 \times 10^6$ CFU per kidney versus $3.2 \times 10^6 \pm 1.2 \times 10^6$ CFU per kidney). Data are presented as the mean number of colonies cultured from 10 mice ± the SEM. One of three separate experiments with the same results is shown.



the growth of *C. albicans* between antibody-treated mice and control mice (Fig. 4). A histological examination of the kidneys from normal and granulocytopenic mice infected with *C. albicans* was performed. Neutrophils were present in fungal infiltrates in the kidneys from normal mice. In the kidneys from granulocytopenic mice, no granulocyte infiltration was seen (Fig. 5). These results are consistent with anticandidal activity of TNF-activated granulocytes in vivo.

Effect of TNF on lethality. Experiments were performed to determine whether the administration of an MAb to TNF would influence the outcome of *C. albicans* infections in mice. No significant difference in lethality was observed between mice treated with the MAb to TNF and control mice after the administration of an LD_{50} of *C. albicans* to either normal or cyclophosphamide-treated mice (Fig. 6). Normal mice were observed for 15 days, and granulocytopenic mice were observed for 8 days, until the restoration of peripheral granulocyte counts.

DISCUSSION

This study shows that TNF and IL-6 are produced during *C. albicans* infections. The differential effect of TN3 19.12 in normal mice and mice with cyclophosphamide-induced granulocytopenia suggests that TNF-activated granulocytes are essential in the normal host defense against infections with *C. albicans*. The effect of TNF may be direct or indirect via secondary induction or suppression of other mediators.

Shalaby et al. demonstrated augmented phagocytotic and cytotoxic activities of human polymorphonuclear cells by TNF and gamma interferon in vitro (27). Furthermore, TNF and gamma interferon have been shown to potentiate the growth-inhibiting activity of human neutrophils against *C. albicans* (5). On the other hand, TNF recently has been reported to reduce the neutrophil killing of *C. albicans* hyphae, despite increased early oxidant responses to hyphal stimulation (4). It is reasonable to relate the TNF-dependent activity against *C. albicans* in our model to granulocytes, as severe granulocytopenia is the dominant effect of cyclophosphamide. However, cyclophosphamide can have other immunomodulatory effects. Available data suggest that cyclophosphamide inhibits preferentially B-cell (26) and suppressor T-cell (21) functions. Irrespective of the mechanisms of cyclophosphamide effects, an important aspect of our model is that it imitates the treatment situation, as patients made granulocytopenic by treatment with cytostatic agents are particularly susceptible to infections with *C. albicans* (2, 14).

Our study is the first report of an enhancing effect of TNF on antimicrobial activity against *C. albicans* in vivo. Riipi and Carlson found no effect of a polyclonal antibody to TNF on the growth of *C. albicans* in mice (24). This result may be

FIG. 5. Histological appearance of periodic acid-Schiff-stained kidney tissue from normal mice (A) and granulocytopenic mice (B) infected with an LD_{50} of *C. albicans*. Tissues were collected from surviving mice 72 h after infection. The histological examination demonstrated that the stage of fungal infiltration was comparable in normal and granulocytopenic mice. Black areas represent fungal infiltration. Granulocytes were present in fungal infiltrates in the kidneys from normal mice (arrows) but not granulocytopenic mice. The darker appearance of the fungal infiltration of kidney tissue from the normal mice than of that of kidney tissue from the granulocytopenic mice was caused by leukocyte infiltration, mainly granulocytes.

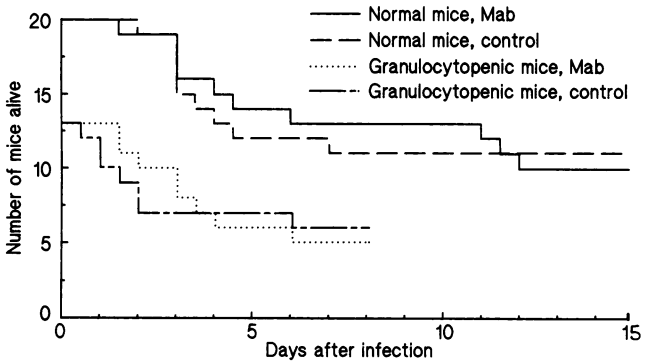


FIG. 6. Lethality during *C. albicans* infections in normal and granulocytopenic mice treated with an MAb to TNF and in normal and granulocytopenic controls. Normal and granulocytopenic mice were infected with 5×10^8 and 1×10^8 CFU of *C. albicans*, respectively. For both the normal and the granulocytopenic mice, no significant difference in lethality was observed between antibody-treated and control mice.

explained by the cultivation of *C. albicans* from the liver and pancreas in their study, as other studies have demonstrated that the kidneys are the most representative organs for the determination of the in vivo growth of *C. albicans* in mice (15, 25). It is reasonable to expect that increased proliferation of *C. albicans* will result in increased numbers of deaths; this relationship has been demonstrated in other experiments as well (15). It may therefore seem surprising that the administration of an MAb to TNF did not significantly influence lethality over that in controls for normal mice. However, the mechanisms by which an overwhelming proliferation of *C. albicans* leads to organ failure and death are not known. TNF is supposed to influence the outcome of serious infections (31, 37), and the role of TNF in the mechanisms leading to death may be other than its role in the antiproliferative effect. Furthermore, other investigators have reported a dissociation between the growth of a microorganism and lethality. Van der Meer et al. (32) demonstrated that IL-1 protects against a lethal infection with *P. aeruginosa* in mice. IL-1-treated mice survived and control mice died with the same numbers of organisms in their organs. The number of organisms in the spleen was even higher in the group showing the lower lethality (32). Our results may indicate that TNF can interfere with the normal relationship between the growth of *C. albicans* and lethality.

The endogenous production and beneficial role of TNF in *C. albicans* infections are very similar to what has previously been demonstrated in *L. monocytogenes* infections (10). The administration of polyclonal immunoglobulin G to TNF to sublethally infected mice resulted in an increased proliferation of organisms in the spleen and liver and ultimately in death from an overwhelming infection (11, 20). Furthermore, the administration of murine recombinant TNF protected mice against a lethal challenge with *L. monocytogenes* (11).

The kinetics of TNF production induced by *C. albicans* infection are obviously different from those induced by endotoxin. The kinetics of the TNF response after the i.p. administration of endotoxin in mice show an abrupt increase in TNF levels, with peak values occurring between 60 and 75 min and then rapidly declining (3, 29a). The distinctly different kinetics of TNF production associated with *C. albicans* and endotoxin exclude the possibility that the TNF production observed in this study was due to contamination with endotoxin.

IL-6 is clearly related to infections; however, its exact role is not yet defined. IL-6 can induce fever in rabbits (13), and it was demonstrated recently that antibody to IL-6 protects against a lethal *Escherichia coli* infection in mice (29). Furthermore, serum IL-6 levels are elevated in septic shock (36), and high levels of IL-6 in spleen homogenates and blood are correlated with an increased proliferation of organisms in infected organs and a fatal outcome in listeriosis in mice (12). Our study demonstrates that IL-6 is produced during infections with *C. albicans*, but at present, the role of IL-6 in these infections remains unknown.

It is conspicuous that TNF production and IL-6 production are higher in cyclophosphamide-treated, granulocytopenic mice than in normal mice, and this difference is not caused by a difference in the proliferation of *C. albicans* in the two groups. This result means that granulocytes are not an important source of TNF and IL-6 during *C. albicans* infections in mice, although human granulocytes have the ability to produce TNF upon *C. albicans* stimulation in vitro (7). Our observation suggests an inhibitory action of granulocytes on TNF production or activity.

The TNF response to stimulation with heat-inactivated *C. albicans* was characterized by early peak levels and rapidly declining levels in serum and was different from the response to an active infection. Furthermore, peak levels were lower than those induced by viable *C. albicans*. After the stimulation of mice with zymosan, a polysaccharide component of the wall of yeast cells, a marked TNF peak occurring after 1 h has been demonstrated (35). This response is different from the response induced in our experiments by heat-inactivated *C. albicans*. Furthermore, the doses of zymosan used were high and were not comparable to the injection of either viable or killed *C. albicans*. The difference between the kinetics induced by viable and heat-inactivated organisms implies that heat inactivation may alter the properties of the fungal wall. TNF-inducing material may be lost during heat treatment, or proliferation may be needed for the late and protracted TNF response. The difference between the kinetics induced by viable and heat-inactivated *C. albicans* indicates that experiments with viable *C. albicans* are required to obtain results relevant to the pathogenesis of infection.

Infections with *C. albicans* are a severe threat to patients with agranulocytosis. These infections respond poorly to antifungal therapy, and the restoration of host resistance is usually necessary for a cure to be achieved (2, 14). In summary, our study demonstrates that both TNF and IL-6 are produced during infections with *C. albicans* and that the beneficial effect of TNF in these infections seems to be dependent on the presence of granulocytes. The therapeutic potential of the administration of TNF-activated granulocytes deserves investigation.

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