INFECTION AND IMMUNITY, Nov. 1996, p. 4561–4566 Vol. 64, No. 11
0019-9567/96/$04.00+0

Intravenous Injection of *Candida*-Derived Mannan Results in Elevated Tumor Necrosis Factor Alpha Levels in Serum

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Received 6 May 1996/Returned for modification 13 June 1996/Accepted 23 August 1996

Intravenous injection of *Candida albicans* into mice produced elevated serum tumor necrosis factor alpha (TNF-α) levels. We hypothesized that immunostimulants released in vivo from *C. albicans* during fungal sepsis might contribute to the elevated levels of TNF-α in serum. We tested this hypothesis in mice with *C. albicans* mannan (CAM). Increased serum TNF-α levels were observed following intravenous and intraperitoneal injections of CAM. Injection of CAM into mice resulted in increased serum TNF-α concentrations that reached 1,200 pg/ml of blood, compared with 2,400 μg/ml of blood following injection of 10 μg of endotoxin. The response to CAM was concentration dependent, requiring a minimum dose of 20 μg of CAM per g of body weight. Sera from mice were tested 30, 60, 90, and 120 min after intravenous injections with CAM. TNF-α concentrations were minimal 30 and 120 min after intravenous injection and maximal 60 and 90 min after CAM injection. The relative distribution of CAM in vivo in decreasing order was determined to be as follows: blood > liver > lung > spleen, 90 min following injection of a single 5-mg dose of CAM. CAM was confirmed as the stimulating substance by utilizing anti-CAM antibodies in vivo to block the response. Rabbit anti-mannan antibodies administered by intraperitoneal injection 24 h before CAM injection significantly suppressed ($P < 0.05$) the accumulation of TNF-α in the sera. Dexamethasone administered to mice before intravenous injection of mannan significantly reduced (40 to 90% reduction; $P < 0.05$) the concentrations of TNF-α in the sera of treated mice. Thus, when in vivo CAM clearance mechanisms are exceeded, sufficient CAM may become available to stimulate TNF-α production, making CAM an important part of pathogenesis in *Candida* sepsis.

Nosocomial infection due to *Candida albicans* is a major cause of morbidity and mortality in hospitalized patients (2, 12, 15, 30). The susceptible patient population includes postsurgical patients, patients with hematologic malignancies, patients undergoing myelosuppressive cytotoxic chemotherapies, and patients with AIDS (17). Most vulnerable are those patients with severe neutropenia regardless of etiology (30). In general, invasive candidiasis is defined by the presence of *Candida* spp. in the blood or deep tissues. However, deep-tissue infections are not always accompanied by positive blood culture of *Candida* spp. As a result, clinical diagnosis may rest solely upon nonspecific observations. Most of these observations, including neutropenia, fever, and right-upper-quadrant tenderness, are suggestive of a systemic inflammatory response. Characterizing the release of cytokine(s) in response to *C. albicans* may provide clinical correlates which explain the physical symptoms noted in the disease involved.

Microbial infections may induce the systemic inflammatory response syndrome (SIRS), a condition that is triggered by the release of interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) by reticuloendothelial cells in response to microbial products. The syndrome can progress to systemic shock, a condition characterized by hypotension, cardiac failure, respiratory distress, leukocytosis, and kidney and liver damage (reviewed in reference 29). The mediators of fungal SIRS are not as well characterized as the mediators of bacterial SIRS. Because the fungal cell wall constituent mannan is released into the blood during *Candida* infections, this substance may be responsible for or influence the induction of cytokines, which then induce SIRS.

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In candidiasis, the candidacidal activity of the immune system coupled with the rapid growth and death of yeast cells within deep tissues may introduce *C. albicans*-derived mannans into the bloodstream (5–7). To an extent, these *Candida*-derived materials are cleared by nonimmune cellular receptors, mannan binding proteins, and antibodies specific for mannans. When these mechanisms are saturated, mannan is free to interact with other ligands such as macrophage (Mϕ) receptors. *Candida* interaction with host tissues and a subsequent increase in monokine (IL-1 and TNF-α) production were demonstrated by studies that used intact *C. albicans* as the stimulant (1, 27, 28). Moreover, the production of TNF-α in response to *C. albicans* has been correlated with the release of at least one acute-phase protein, fibrinogen (23).

C. albicans mannan (CAM) accumulates in the blood during systemic spread of *Candida* infection (5). *C. albicans* and free CAM readily bind to polymorphonuclear leukocytes (PMN) and Mϕ. Therefore, hematogenous dissemination of CAM from infected tissues increases the potential number of phagocytes that might bind to and respond to CAM. Of particular interest is the response of tissue Mϕ, which may secrete variable combinations of immunomodulatory monokines when exposed to microbes or microbial products, to CAM (10, 14, 25, 29). Because TNF-α is produced principally by cells of the mononuclear-phagocyte system, information pertaining to the accumulation of this cytokine in the sera of mice injected with CAM may shed light onto the systemic response of these cells to CAM during candidiasis.

We have shown previously (14) that Mϕ produce TNF-α in response to CAM and have formulated the hypothesis that CAM administered in vivo will lead to the systemic release of TNF-α. We show here that the injection of mice with CAM

results in increased serum TNF-α concentrations. We describe the kinetics and concentration dependency of the response to CAM. Furthermore, we demonstrate that the production of TNF-α in response to CAM can be controlled in mice by specific anti-CAM humoral immunity and oral corticosteroid treatment.

MATERIALS AND METHODS

Animals. Female ICR mice (6 to 8 weeks old) from Harlan Sprague-Dawley, Indianapolis, Ind., were maintained under standard laboratory conditions, with food and water available ad libitum. The protocol used in this study was approved by the Mercer University Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines for the humane use of experimental animals.

Organism and culture conditions. *C. albicans* 20A was a gift from Judith E. Domer, Tulane University Medical School, New Orleans, La. Cultures were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants at 24°C. Fresh transfers were made into 100 ml of tryptic soy broth (Difco) plus 0.5 M glucose, and the pH was adjusted to 6.9. Liquid cultures were incubated at 37°C in an Orbit incubator-shaker (model 3526; Lab-Line Instruments, Inc., Melrose Park, Ill.) for 18 h.

Isolation and purification of fungal and bacterial substances. Mannan was extracted from *C. albicans* 20A that was grown overnight in tryptic soy broth with 0.5 M glucose. The culture was incubated for 24 h at 37°C in a fermentor (Biostat E; B. Braun, Allentown, Pa.) under continuous aeration and agitation. Production of CAM from cultured yeast cells has been described previously (9, 20). Briefly, a culture of *C. albicans* was washed three times in ice-cold phosphate-buffered saline (PBS), pH 7.2, before extraction in hot citrate buffer (9). The crude extract was dialyzed against distilled water and lyophilized. The citrate-extracted polysaccharide was further purified by hexadecyltrimethylammonium bromide (CTAB) precipitation (22), hereafter referred to as CTAB-CAM. Bacterial lipopolysaccharide (LPS; *Salmonella typhimurium*, lot 114F-4041) was purchased from Sigma Chemical Co., St. Louis, Mo.

Mannan and LPS were used at the concentrations indicated in each individual experiment. These stimulants were solubilized in ice-cold Hanks' balanced salt solution (HBSS; Fisher Scientific Co., Atlanta, Ga.). The following precautions were taken to eliminate exogenous LPS contamination in these experiments. Glassware was washed and rinsed with deionized, double-distilled water which was tested by *Limulus* assay and found to be free of endotoxin. All glassware was sterilized by baking at 200°C for 3 h.

Despite our efforts to store CAM and preserve its activity, we still observed a decrease in its activity suggestive of a 30-day shelf half-life. Taken together, the dose requirement and lability of the CAM suggested that the active component was unstable. This necessitated continual testing of lots of the CAM and required that only fresh CAM be used for analysis. This observation of CAM's sensitivity to storage demonstrated one more CAM feature that was distinctly different from those of endotoxin.

CAM injection. CAM used for injection was stored lyophilized in amber glass vials at −20°C. CAM preparations were discarded after 12 months or if a loss in activity was noted in cultures of Mϕ (14). CAM preparations were compared for stimulatory activity in an in vitro Mϕ culture model and were found to be equivalent (14). Only CTAB-CAM was used in this study. Only preparations which were tested and found to be negative by *Limulus* assay (Sigma Chemical Co.) were used. Separate experimental groups have been tested with mannan prepared by Cu precipitation or CTAB precipitation (14) and when freshly prepared no differences were noted. In vivo experiments were performed with groups containing at least five mice. Controls were injected with HBSS. Enough CTAB-CAM was dissolved into 5 ml of HBSS to yield a 500-μl injection with the desired amount of CAM, which was injected into the tail vein of each animal. For example, 25-g ICR mice were injected intravenously (i.v.) with an optimal dose of 40 μg of CAM per g of body weight, in a total volume of 500 μl of HBSS.

Analysis of TNF-α and CAM. Blood was drawn from mice by cardiac puncture, and the serum separated with pediatric serum separation tubes (Becton Dickinson, Inc., Rutherford, N.J.). The sera were stored at −70°C until needed. TNF-α was measured in the sera with a commercial enzyme-linked immunosorbent assay (ELISA) kit purchased from Genzyme (Cambridge, Mass.), and TNF-α activity was measured by assessing the cytotoxicity of samples in the TNF-α-sensitive cell line L929. Procedures for L929 cytotoxicity assays are described elsewhere (14). The TNF-α ELISA uses a monoclonal antibody specific for TNF-α as a capture reagent and a polyclonal anti-TNF-α antibody as a detector. Concentrations of TNF-α are determined by comparison with known concentrations of recombinant murine TNF-α (Genzyme). The system is sensitive to picogram concentrations of TNF-α. ELISA results were confirmed by measuring TNF-α activity by the bioassay method, a technique which uses TNF-α-sensitive L929 target cells.

A CAM ELISA which uses human ligands specific for CAM to capture CAM was developed. Bound CAM is detected with purified rabbit anti-mannan antibodies (DAKO Carpinteria, Calif.). The human sera with anti-CAM antibodies are obtained from human volunteers, pooled, and stored. The pooled sera are diluted 1:500 into coating buffer (PBS with 0.05% NaN_3; PBSN), and 200 μl of the solution is added to each well of a Dynatech Immunlon I (Dynatech Laboratories, Chantilly, Va.) 96-well plate. After a 1-h incubation at room temperature, the plate is washed with PBS-Tween 20 (PBST) and blocked with 1% bovine serum albumin in PBSN. The plates are then washed with PBST (3×) and subsequently incubated with mouse sera containing CAM for 1 h. After this incubation the plates are rinsed four times with wash buffer (PBST) and incubated with rabbit anti-CAM (DAKO) for 1 h at room temperature. The last incubation is with peroxidase-linked goat anti-rabbit antibody (DAKO; used at a 1:1,000 dilution in PBS with 0.1% BSA). Following a 1-h incubation, goat anti-rabbit antibody is removed and the plate is rinsed with PBST three times. ELISA reactivity is visualized by using a standard peroxidase-ABTS (Sigma Co.) reagent cocktail. The tissue CAM values were obtained by interpreting x values from a linear standard curve generated with dilutions of CAM (including a negative control with no CAM) where the data yielded a linear regression with an r^2 of 0.994.

Statistics. The procedure for interpretation of the ELISA results is based on a regression analysis such that duplicate samples are removed from each animal and compared with dilutions of a TNF-α standard. Sample means for each animal are averaged to yield a population mean for a group of animals (n = four to six mice). To determine significant differences between mannan-treated and saline-treated animals, Student's t test was employed (significance was noted when P was <0.05), and significance is denoted in each figure in this article by a single asterisk. For multiple group comparisons, a two-way analysis of variance (ANOVA) was employed and the significance ($P < 0.05$) is noted in each figure with double asterisks.

RESULTS

Serum TNF-α levels in mice given i.v. injections of *C. albicans*. In our initial studies, we examined the response of mice to *C. albicans*. Sixty to 90 min following i.v. injection of live *Candida* organisms, the sera from ICR mice possessed elevated TNF-α concentrations. Injections of 10^6 and 10^8 live *Candida* organisms both induced significant amounts of TNF-α in vivo (Student's t test; $P < 0.05$). When 10^6 *Candida* organisms was given by i.v. injection, approximately 270 pg of TNF-α per ml of sera was detected 2 h after the *Candida* injection. Serum TNF-α levels increased significantly (ANOVA; $P < 0.05$) during this time course to yield 650 pg/ml of blood at the 8-h time point. Beyond 8 h animal death precluded accurate TNF-α measurement.

Kinetics of serum TNF-α accumulation after CAM treatment. Each mouse received an injection of CAM either i.v. or i.p. equivalent to 40 μg/g of body weight. Controls received nonpyrogenic HBSS. When TNF-α was measured in sera 6, 12, 18, and 24 h after CAM injection, detectable levels were not found. However, when TNF-α in serum was measured at shorter periods after CAM injection, i.e., 30, 60, 90, 120, and 240 min, serum TNF-α levels were significantly greater ($P < 0.05$) than those detected in normal mouse serum. The highest serum TNF-α values were observed 60 to 120 min following injection of 40 μg of CAM per g of mouse body weight (Fig. 1, EXP. A and EXP. B). The lower serum values shown in Fig. 1 at the 30-min time point (EXP. A) are partially due to the fact that only 40% of the animals were found to have detectable TNF-α levels in their blood at this time point, whereas by the 60-min time point (EXP. A) 100% of the animals injected with CAM demonstrated elevated TNF-α levels. On the basis of a multigroup analysis (Freidman ANOVA) which utilizes rank order testing, the optimal response time was 60 min (significance indicated if P was <0.05). In three different experiments (4 h and daily for up to 14 days) we noted that the TNF-α response was not detectable later than 4 h after i.v. injection of CAM. The effects of CAM in vivo were not restricted by route of injection, since intraperitoneal (i.p.) injection of similar amounts of CAM (Fig. 1, EXP. B) per animal also resulted in significantly elevated serum TNF-α levels (Student's t test; $P < 0.05$) 60 and 90 min after CAM injection. The optimal response was noted following maximal TNF-α accumulation (Fig. 1, EXP. B) 90 min after i.p. injection of CAM (ANOVA; $P < 0.05$).

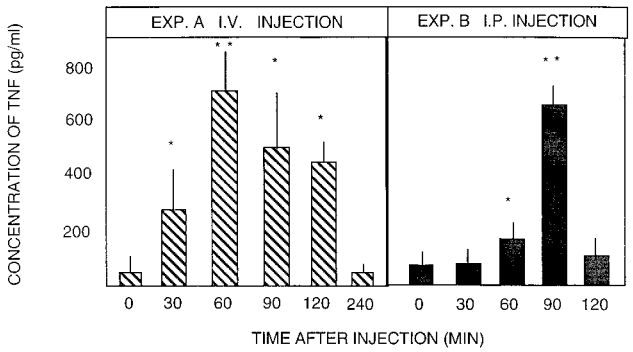

FIG. 1. (EXP. A) TNF-α levels that are found in the blood of ICR mice which are injected i.v. with 40 μg of CAM per g of body weight in a volume of 200 μl of nonpyrogenic saline. Mice were bled by cardiac puncture 0, 30, 60, 90, and 120 min after i.v. injection of CAM. (EXP. B) TNF-α values from animals injected i.p. with CAM. Control animals were injected with saline and examined at each of the indicated time points. All of the control values were below detectable limits. Values represent the means and standard error of the mean (SEM) of duplicate samples taken from four to six mice. Significant differences between each experimental group and the control group were tested by Student's *t* test, and significance ($P < 0.05$) is denoted by a single asterisk. Significant differences between a particular group and the rest of the test groups as a whole population were identified by ANOVA, and significance ($P < 0.05$) is identified by double asterisks.

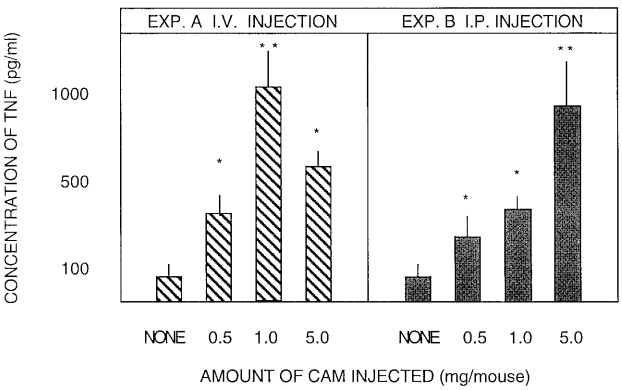

FIG. 2. Various doses of CAM were administered i.v. (EXP. A) or i.p. (EXP. B) to ICR mice, and the sera were evaluated for TNF-α 60 min after CAM injection. The CAM used here was derived from the CTAB procedure. Doses of 500, 1,000, and 5,000 μg of CAM were administered to ICR mice (average weight = 25 g). The mice were bled by cardiac puncture, and duplicate samples were evaluated for the TNF-α levels in their sera by ELISA. Values represent the means and SEM of duplicate samples taken from four to six mice. Significant differences between each experimental group and the control group were tested by Student's *t* test, and significance ($P < 0.05$) is denoted by a single asterisk. Significant differences between a particular group and the rest of the test groups as a whole population were identified by a two-way ANOVA, and significance ($P < 0.05$) is identified by double asterisks.

Dosage of CAM required to induce detectable amounts of TNF-α in mouse sera. A number of CAM-binding ligands exist in vivo that may influence the in vivo response to CAM (26, 31). Some of these ligands may enhance the response, whereas others may interfere with it. In this context, the dosage of CAM used may be directly related to the saturation of one or more of these ligands. We have also noted in vitro that peritoneal macrophages do not respond to CAM, whereas alveolar macrophages do respond. To further address this feature of the response to CAM in vivo and to determine the optimal doses of CAM required to stimulate mice, we administered CAM by either i.p. or i.v. injection. Comparison of different CAM doses demonstrates that i.v. injection of as little as 500 μg per mouse (20 μg of CAM per g of body weight) induced the release of TNF-α into the sera (Fig. 2, EXP. A and B). When mice were injected i.p. with large doses of CAM (Fig. 2, EXP. B; 5 mg of CAM per animal; 200 μg of CAM per g of body weight), significantly higher (ANOVA; $P < 0.05$) TNF-α levels were observed in the blood than when lower doses were administered i.p. (Fig. 2, EXP. B; 0.5- and 1.0-mg doses). This is in contrast to TNF-α induced by i.v. injection, where a dose of 1 mg of CAM per animal yielded significantly higher (ANOVA; $P < 0.05$) serum TNF-α levels than did the 5- and 0.5-mg doses (Fig. 1, EXP. A). The TNF-α data from the i.p. and i.v. experiments were determined by using the same CAM and the same TNF-α standards, which allows some comparisons to be made. When serum TNF-α values from i.v. and i.p. injections are compared, i.v. injection with 1 mg of CAM was found to be the optimal course of treatment. Doses of 0.1 mg or less of CAM were administered to mice in three separate experiments and were found to yield <50 pg/ml of TNF-α (data not shown).

Evaluation of CAM in circulation after i.v. injection. While in the serum, CAM is probably bound by mannose binding proteins (26, 31). Once these ligands are saturated, all additional unbound mannan is available to stimulate cells which possess mannan receptors. A study by Kappe and Muller (16) addressed the point of CAM clearance by using CAM in BALB/c mice. The authors reported that CAM was rapidly removed by the liver and spleen. The half-life of CAM in the sera was shown to be 2 h. During the course of these experiments the concentration of CAM was reduced to levels of <200 pg/ml of sera 18 h after i.v. injection of 20 mg of CAM. In particular, CAM persisted in the liver for 97 days. We used a capture ELISA (described in Materials and Methods) to detect CAM in the sera of mice that were injected i.v. with 5 mg of CAM per animal. In our hands, blood obtained from animals injected i.v. with 5 mg of CAM contained approximately 50 μg of available CAM per ml of blood 90 min after injection. If each mouse contained at least 4 ml of blood, then only 1% of the injected CAM remaining in the serum was accessible to the ELISA antibodies 90 min after injection. Since the detection method employed here used an anti-CAM antibody, saturation of CAM with nonimmune ligands in vivo could reduce the number of epitopes which are available to interact with the capture or the detection antibody. The concentration of CAM used was 5 mg, an amount that should not be saturated in vivo by the binding of nonimmune ligands. Therefore the accumulation of CAM in tissues probably represents an active binding process that parallels the release of TNF-α into circulation. As a result of decreasing serum CAM concentrations, CAM may decrease to levels that are insufficient for stimulation, possibly explaining why the response to CAM decreases between 90 and 120 min. Moreover, the relative distribution of CAM in vivo 90 min following injection of a single 5-mg dose of CAM was determined to be as follows: blood > liver > lung > spleen. This agrees with the observations of Kappe and Miller, who noted that the liver is the primary clearance organ for mannan.

Blockade of TNF-α secretion in vivo by rabbit anti-CAM. A number of mannan ligands are present in mammalian tissue (5, 16, 26, 31). Since a portion of these ligands are free in the serum or on cells which do not produce TNF-α, they may represent antagonists to CAM-induced TNF-α secretion by binding CAM in vivo and preventing it from interacting with ligands on responsive cells. Naive mice lack antibodies specific for mannan. Humans, on the other hand, possess detectable anti-mannans. Therefore, we hypothesized that the addition of

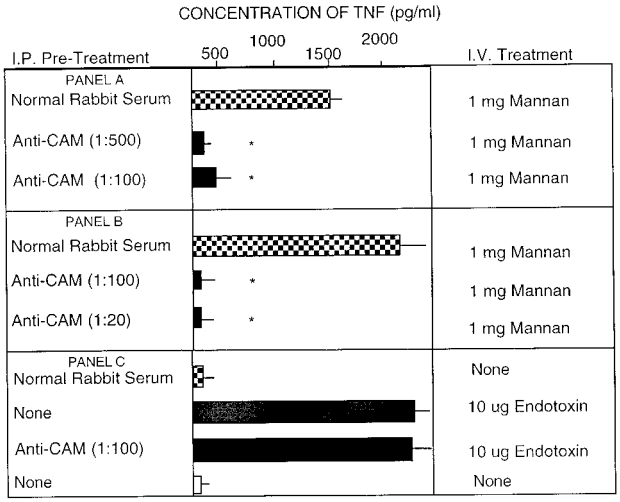

FIG. 3. Rabbit anti-CAM antibodies were used to passively immunize mice versus CAM. Panels A through C represent three different experiments. One-half milliliter of the listed dilutions of DAKO anti-mannan antibody or pooled normal rabbit sera was given to mice 24 h prior to CAM challenge (left side of panels). The next day mice received an i.v. injection of a microbial stimulant as noted on the right side of the graph. The mice were bled by cardiac puncture, and the TNF-α concentration in their sera was determined by an L929 bioassay (A) or ELISA (B and C). Values represent the means and SEM of duplicate samples taken from four to six mice. Significant decreases in each experiment were identified by comparison to the group that received normal rabbit sera (where P was <0.05) by a two-way ANOVA and are indicated by double asterisks.

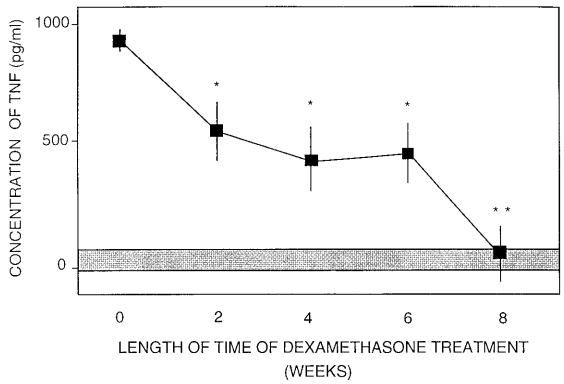

FIG. 4. Dexamethasone was administered to mice in their drinking water at a concentration of 2 μg/ml. The concentrations of TNF-α were measured in the sera of these mice after they were given an i.v. injection containing 1 mg of CAM. The values shown are averages of the values determined from groups containing five mice per time point (solid boxes). The shaded horizontal bar represents the mean and SEM of TNF-α measured in the sera of dexamethasone-treated mice that were injected with normal nonpyrogenic HBSS. All values represent the means and SEM of duplicate samples taken from four to six mice. Significance differences were determined by Student's t test (single asterisk) between dexamethasone-treated CAM-stimulated mice and nondexamethasone-treated CAM-stimulated mice. The most significant group (where $P < 0.05$) was identified by a multigroup ANOVA and is indicated by double asterisks.

a ligand, such as anti-mannan immunoglobulin, to naive mice would decrease the stimulatory action of CAM by preventing its interaction with CAM-responsive TNF-α-producing cells. To test this hypothesis, we passively immunized mice with rabbit anti-CAM and measured the TNF-α response to CAM using an L929 bioassay (Fig. 3A) and using an ELISA (Fig. 3B and C). ICR mice were injected with dilutions of rabbit anti-CAM (DAKO) or normal immunoglobulin (Ig) 24 h prior to CAM injection. Since anti-CAM was positive in a *Limulus* assay, a chance that contaminating endotoxin could desensitize the mice existed. To account for this possibility we treated several groups of mice with anti-CAM and then stimulated the mice with endotoxin in the same manner that the CAM stimulation was to be carried out (Fig. 3C). Anti-CAM failed to inhibit endotoxin induction of TNF-α in vivo. Treatment of mice with either LPS alone or anti-CAM plus LPS yielded 2,500 pg of TNF-α per ml of serum. Alternatively, anti-CAM rendered mice unresponsive to CAM, as determined by a lack of TNF-α in serum following CAM stimulation. Mice which received normal rabbit serum (IgG) and CAM produced 1,500 to 2,200 pg/ml of TNF-α, whereas mice which received anti-CAM prior to CAM injection displayed a significant (ANOVA; $P < 0.05$) 60 to 90% reduction in serum TNF-α. The possibility that normal rabbit sera might be stimulatory was dismissed when blood from test groups given normal rabbit sera alone was found to be negative for TNF-α (Fig. 3C).

Reduction of in vivo responsiveness by dexamethasone treatment. Dexamethasone pretreatment of mice reduces their cytokine (serum IL-1 and serum TNF-α) response to endotoxin treatment and therefore supports the use of this approach in septic shock therapy (21). Because the mechanism of this intervention alters the cytokine cascade at the transcriptional and translational levels of cytokine production, it seemed that this effect might function independent of the stimulus (LPS versus CAM) that was used. Therefore, dexamethasone treatment could desensitize the hosts, making them unable to release TNF-α into the serum in response to CAM injection. In pursuit of this hypothesis, mice were given dexamethasone in their drinking water (2.4 μg/ml) and tested for their sensitivity to CAM. CAM was administered at 40 μg/g of body weight and TNF-α was measured 1 h after i.v. CAM injection. As shown in Fig. 4, sensitivity to CAM was reduced by dexamethasone treatment in comparison with the 0 time point. Dexamethasone treatment significantly reduced TNF-α levels by 45% ($P < 0.05$) as early as 2 weeks following the initiation of dexamethasone treatment and continued at this level for 5 weeks. Multigroup analysis by ANOVA ($P < 0.05$) showed that the greatest suppression of TNF-α in serum was correlated with 8 weeks of dexamethasone treatment. At this point the degree of suppression increased to a level of >90%, where TNF-α levels in the sera of treated mice were indistinguishable from the TNF-α levels in the sera of nontreated control mice.

DISCUSSION

The primary objective of our study was to demonstrate that CAM participates in the release of TNF-α in vivo. Our working hypothesis was that *C. albicans* induces TNF-α in vivo via a mannan specific mechanism. In vivo yeast cells or their derivatives may stimulate blood monocytes, PMN, tissue Mϕ, and natural killer cells to produce TNF-α (23, 28). This action in turn enhances the candidacidal activity of human monocytes and PMN (3, 4, 13). Live *C. albicans* (23, 27, 28) or CAM (14, 19, 28) has been used to study the relationship of TNF-α with *Candida* immunity. Vecchiarelli et al. (28) demonstrated the ability of CAM to stimulate leukocytes and showed that mannoprotein produced by a chloroform-butanol procedure stimulated splenocyte cultures (lymphocytes plus adherent cells). Both TNF-α and gamma interferon were found in the splenocyte cultures and appeared to have a regulatory role. Others showed that CAM could induce altered blood flow in rats (19). Collectively, these reports support our hypothesis regarding the role of CAM in the TNF-α response observed during

candidiasis and prompted us to investigate the in vivo secretion of TNF-α into the blood after injection of CAM. In the present study, we report that i.v. injection with *C. albicans* or CAM elicits detectable serum TNF-α levels.

CAM that circulates in vivo during infection is derived from the cell wall of *C. albicans.* The cell wall of *C. albicans* contains a small amount of chitin, some proteins, glycopeptide, beta-glucan, and mannan (22). On a dry-weight basis, CAM is the most abundant cell wall component in yeast cells and blastospores. CAM is a glycopeptide with an alpha-(1→6) mannosyl backbone or core. Branching, heavily substituted oligosaccharide side chains of alpha-(1→2)-linked and alpha-(1→3)-linked mannosyl branches are attached to the core alpha-(1→6) polymer. The alpha-(1→6) backbone polymer is attached to a peptide that accounts for approximately 10% of the molecular weight of the CAM molecule (9, 22). CAM is an immunostimulatory molecule, and its varied interactions with the immune system have been reviewed previously (22).

Bloodstream clearance of *C. albicans* results in the adherence of yeast cells within the liver, lungs, and spleen. Liver clearance of *C. albicans* from the bloodstream by endothelial trapping has been studied in some detail, and it has been shown previously that CAM may facilitate yeast cell adherence to hepatic sinusoidal endothelial and Kupffer cells (24). In comparison, other studies have found that the interaction between *C. albicans* and the lungs may play a significant role in sepsis (17). This suggests that the capillary beds of the lungs may also contribute to the clearance of *C. albicans*. Soluble CAM, however, may not be cleared (or trapped) as efficiently by the lungs as it is by the liver or spleen, since the liver mechanism is clearly receptor mediated and shows affinity for mannose polymers (26, 31). Kappe and Muller examined the kinetics of CAM circulation in mice and found that 50% of the injected CAM is removed from circulation 2 h after injection. Their study as well as the present study indicates that CAM accumulates predominantly in the liver and the spleen, compared with the lungs. Thus, the reticuloendothelial system in these organs may play an important role in the systemic response to *Candida* spp. by removing CAM from and releasing cytokines into the blood.

In our initial time course experiments, mice responded optimally 60 min after i.v. injection of CAM. Mice which were given an i.p. injection of CAM reached similar levels of responsiveness at 90 min after CAM injection. We also noted a peak response time of 8 h for mice that received live *Candida* organisms by i.v. injection. Steinshamn and Waage (27) reported that injection of live *C. albicans* does not induce significant TNF-α levels in mice. We observed, however, that live or killed *C. albicans* induces TNF-α when injected i.v. Perhaps the differences in our observations are due to variable efficiencies in the delivery of *C. albicans. C. albicans* given by i.v. injection gains access to the responding cells, whereas i.p.-injected *C. albicans* never reaches the responding cells in quantities sufficient to cause stimulation. To stimulate the response in vivo, the competitive nonstimulatory ligands may have to be saturated so as to allow sufficient stimulation of the stimulatory ligands. A number of ligands that bind mannose polymers exist in vivo, evidenced by the rapid removal of mannan from circulation (16). We expect that these ligands include at least two types, those which facilitate the TNF-α response and those which by virtue of being nonstimulatory are antagonistic to the stimulatory process. Therefore, the effect of CAM might be dependent on overcoming a stimulatory threshold. We observed that the minimal stimulatory dose was approximately 20 μg of CAM per g of mouse body weight.

TNF-α accumulation in the serum agreed with the kinetics of CAM removal which had been noted previously by Reiss and coworkers (22). They showed that during *C. albicans* infection two forms of CAM circulate in the serum, uncomplexed (free) CAM and CAM complexed with Ig or mannose-binding serum proteins in the serum. In the current study, >90% of the CAM which was injected into mice was removed from the blood 90 min after injection. The decreasing order of localization for CAM, on the basis of ELISA analysis of tissue, was as follows: blood > liver > lung > spleen. Unlike adult humans, adult mice do not possess anti-CAM antibodies. Thus we presume that the removal of CAM by these aforementioned organs is most likely via mannose-specific ligands.

Antibodies to CAM are commonly observed in human sera (22). This feature of human anti-*Candida* immunity differs from the innate mechanisms of immunity demonstrated by mice. In humans, CAMs that are present in the serum are likely bound into immune complexes that probably involve complement. These immune complexes can be adsorbed to a number of cells which possess the proper receptors. Cells with C3b receptors such as erythrocytes, monocytes, Mϕ, PMN, and NK cells can serve as shuttles for CAM-immune complexes to the liver, spleen, or kidney. However, CAM that is removed in this way may be unavailable to stimulate Mϕ. In our in vitro study (14) it appeared that serum factors were not necessary for stimulation of Mϕ with CAM. Perhaps these ligands serve a protective function. During human candidiasis, anti-CAM antibodies may become saturated and free CAM may interact with resident Mϕ populations situated within deep host tissues. Anti-CAM antibodies may participate in the anti-*Candida* response by binding CAM and preventing its interaction with cell-bound ligands which control cytokine secretion. If this hypothesis is correct, the loss of serological response to CAM may render a person susceptible to CAM-induced SIRS.

Our earlier study showed that CAM binding to Mϕ mannose receptors may precede production of cytokines. In that study we used anti-CAM to block the stimulation of Mϕ. In addition to demonstrating the specificity of the in vivo response, the blockage by anti-CAM showed that humoral immunity to CAM could block stimulation and the subsequent release of TNF-α. Since mice are serologically naive to CAM, passive immunization with anti-*Candida* antibody may simulate the human condition. In this study we observed that transfer of rabbit anti-CAM to mice rendered the animals nonresponsive to CAM but did not alter their response to endotoxin. Other features of the reagents that might have contributed to this finding were considered: undiluted rabbit anti-CAM was positive in *Limulus* assays and contained micromolar concentrations of NaN_3 at the dilutions used in this study. The normal rabbit Ig controls lacked these contaminants. Additional data, however, suggested that reagent contaminants did not influence the experimental results. Had the preservative been a factor in stimulation, then anti-CAM would have blocked both CAM and endotoxin stimulation. By showing that only CAM stimulation was affected by anti-CAM, we eliminated the possibility of influence by the preservative or contaminating endotoxin.

In addition to its sensitivity to anti-CAM antibodies, the response to CAM was also modified by corticosteroid. This feature of the TNF-α response to endotoxin is known (21, 29) but has not been correlated with *Candida* responses. Corticosteroids are often administered to patients who are at risk for candidiasis. The interference of these substances with the mechanisms that lead to TNF-α secretion in endotoxin-stimulated Mϕs is associated with intracellular control of transcription and translation. The observation that CAM stimulation in vivo is modified, much like endotoxin stimulation, suggests that

the two mechanisms of TNF-α release utilize a common intracellular stimulation pathway.

In conclusion, we have demonstrated that the cytokine TNF-α is produced in vivo in response to CAM. Moreover, the response can be regulated by mannan ligands, such as anti-mannan antibodies and corticosteroids. In this context, CAM appears to be a potent inflammatory mediator which most likely elicits innate host defenses, but the inflammatory response may be curtailed in a host with humoral immunity.

ACKNOWLEDGMENTS

We gratefully acknowledge Jonathan Martin, Anna Walker, and Jie Tang in the preparation of the manuscript.

This study was supported by grant 16121-50 from the Medical Center of Central Georgia and in part by the Rubye Ryle Smith Trust.

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Editor: D. H. Howard