Release of Tumor Necrosis Factor by Human Polymorphonuclear Leukocytes

By Julie Y. Djeu, Debbie Serbousek, and D. Kay Blanchard

Evidence is presented that human polymorphonuclear neutrophils (PMN) can be induced to produce tumor necrosis factor (TNF). Other investigators have previously reported that TNF has been induced from macrophages by bacteria and, more recently, from natural killer cells by certain tumor cells. Our laboratory has reported that the opportunistic fungi, Candida albicans, can induce TNF, not only from human monocytes, but also from Percoll-fractionated large granular lymphocytes. We now report that incubation of PMN with C. albicans for 3 hours was sufficient for detection of TNF release, and peak observation was achieved at 8 to 18 hours. This release was inhibitable by actinomycin D, an inhibitor of RNA synthesis, as well as by emetine and cycloheximide, which block protein synthesis. The TNF produced by PMN was neutralized by specific monoclonal antibodies against human TNF. These results represent an important finding that TNF production is a normal response of PMN to stimulation by fungi such as C. albicans and suggest that the release of TNF may be related to autocrine activation of PMN effector function to control Candida growth.

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MATERIALS AND METHODS

Preparation of human PMN. Leukocyte buffy coats obtained from normal volunteers at the South West Florida Blood Bank (Tampa, FL) were diluted 1:2 in phosphate-buffered saline (PBS) and layered on 10 mL of Ficoll-Hypaque solution (Pharmacia Fine Chemicals, Piscataway, NJ). After centrifugation at 400g for 30 minutes at room temperature, the WBC layer lying on the surface of the red blood cell pellet was collected and lysed of erythrocytes by hypotonic shock with sterile distilled water for 30 seconds at room temperature. The cells were washed twice in PBS before readjusting to the desired concentration. Cytocentrifuged preparations of PMN stained with Giemsa showed greater than 99% PMN by morphology.

Activation by P. interpertussis.

PMN were suspended in RPMI 1640 media containing 2% fetal calf serum with 100 U/mL of penicillin/streptomycin and were diluted to 1 x 10^6/mL. From the Department of Medical Microbiology and Immunology, University of South Florida, College of Medicine, Tampa. Submitted January 24, 1990; accepted June 11, 1990.

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cells/mL in the wells of a 24-well Costar microtiter plate containing 1 x 10^5/mL of heat-killed *C. albicans*, killed in boiling water for 1 hour. After stimulation with killed Candida for 18 hours at 37°C, the supernatants from the PMN were collected and spun free of cells and debris. Experiments requiring various doses of *C. albicans* for incubation or various times of incubation are detailed in the Results section. Activation with LPS was performed under the same conditions except that serial 10-fold dilutions of *E. coli* LPS 011:B4 (Sigma Chemical Co) were used for stimulation instead of Candida. For inhibition of RNA synthesis, PMN (2 x 10^6/mL) were incubated with 4 μg/mL of actinomycin D, and for inhibition of protein synthesis, with 10^-3 mol/L or 10^-6 mol/L of emetine, as well as with 200 μg/mL or 50 μg/mL of cycloheximide. These concentrations are within the range routinely used for inhibition of metabolism in cells. All the metabolic inhibitors were purchased from Sigma Chemical Co. 

**Titration of TNF.** The usual method for titration of TNF was by the measurement of the lysis of a TNF-sensitive murine cell line, WEHI-164. Cells from an exponential growth phase of the WEHI-164 cell culture were pelleted in a 5-mL sterile plastic tube, and 100 μCi of 51Cr (sodium chromate, Na51CrO4, Amersham, Arlington Heights, IL) was added for 1 hour at 37°C. The cells were then washed three times and adjusted to 5 x 10^6 cells/mL. To 100 μL of the serial dilution of TNF-containing culture supernatants, 100 μL of the labeled WEHI-164 tumor cells were then added. After 18 hours at 37°C, the 51Cr released in the supernatant from lysed target cells was harvested and counted in a gamma counter. Maximum isotope incorporated was determined by counting target cells alone, and spontaneous release was measured by counting supernatants of targets incubated with medium alone. The spontaneous release in the 18-hour assay was in the range of 18% to 25%. The titration was expressed as the reciprocal of the dilution of the sample that caused 20% maximal lysis of WEHI-164 cells and was standardized with recombinant human TNF.

**Neutralization of PMN supernatants with antibodies against TNF.** PMN supernatants, obtained after 18-hour stimulation with heat-killed *C. albicans*, were mixed with an equal volume of medium with or without 1 x 10^7 neutralizing units/mL of monoclonal antibodies (MoAbs) against human TNF (kindly provided by Genentech Inc, San Francisco, CA). After incubation with the MoAb for 30 minutes at room temperature, the supernatants were serially diluted and added to 51Cr-labeled WEHI-164 tumor cells for titration of residual TNF. Recombinant TNF, kindly provided by Genentech Inc, was used as an internal control to check for the efficiency of the MoAb. The MoAb against lymphotoxin (LT) from Genentech Inc, was also used, with recombinant LT as the control.

**RESULTS**

**TNF induction from PMN of normal donors.** To determine if human PMN could release TNF, PMN free of
monocytes and lymphocytes, by morphology (Fig 1) and FACS analysis (Fig 2), were individually processed from 10 normal blood donors and cultured with \textit{C} \textit{albicans} at a stimulator/responder ratio of 10/1. After 18-hour incubation at 37°C, the supernatants were collected and assessed for TNF activity. As seen in Fig 3, PMN from every donor were able to release TNF in response to the stimulant. The levels ranged from 8.1 U/mL to 36.0 U/mL. No TNF was detected in PMN supernatants in the absence of stimulant (ie, 0/1 stimulant/responder ratio).

\textit{Dose response to} \textit{C} \textit{albicans}. Because the amount of stimulus may influence the level of TNF induction, PMN from a normal donor was incubated with \textit{C} \textit{albicans} at a stimulator/responder ratio of 1/1 to 100/1 (Fig 4). After incubation at 2 or 18 hours at 37°C, TNF levels were measured in the supernatants of the cells. With 2 hours of incubation, a slow increase in TNF production was observed that correlated with increasing numbers of stimulating fungi. Incubation for 18 hours with \textit{C} \textit{albicans} did not change PMN viability as assessed by trypan blue exclusion but caused a dramatic increase in TNF levels, with the highest levels induced between 10/1 and 30/1 stimulator/responder ratios.

\textit{Time kinetics of induction of TNF}. A more detailed analysis of the kinetics of TNF induction was performed next. Normal PMN, after addition of \textit{C} \textit{albicans}, were cultured for 1/2, 1, 2, 3, 8, or 18 hours at 37°C before assessment of TNF (Fig 5). Within the first 2 hours of incubation with \textit{C} \textit{albicans}, PMN did not release appreciable levels of TNF. At 8 hours, however, a significant level of TNF was detected that was still on the rise at 18 hours. In the absence of \textit{C} \textit{albicans}, no TNF was detected at all the time points tested.

\textit{Requirement for transcription and protein synthesis in TNF release from PMN}. The previously mentioned kinetics indicating a minimum of 8 hours for optimal TNF release from PMN suggested that some protein synthesis was required for production of TNF. To directly assess this phenomenon, PMN were treated with actinomycin D, an inhibitor of RNA synthesis, or with emetine or cycloheximide, which both block protein synthesis (Fig 6). All the antimetabolites, at the doses used, had no effect on PMN viability by trypan blue exclusion, but did have a profound inhibition of TNF release from PMN. These results suggest that new transcription and translation occur in PMN responding to \textit{C} \textit{albicans} with the release of TNF.

\textit{Induction of TNF by LPS}. To examine if a soluble antigen, rather than a particulate organism, can induce TNF from PMN, normal PMN were incubated for 18 hours at 37°C with LPS over a wide range of concentrations, and the supernatants were assessed for the presence of TNF. Figure
Neutrophils have been extensively studied and widely accepted as the key effector cells in host defense against fungi and other microbes. This has led to the search for means to activate these cells for better understanding of the control of these cells or for possible application in immunotherapy. Our studies have confirmed the importance of PMN as the end effector cells against the opportunistic fungi *C. albicans*, and we have further evidence that TNF is a key activator of PMN function. Less than 1 U/mL of TNF was capable of significant activation of PMN, and this response occurred within 2 hours of incubation with the cytokine. In the present study, we provide definitive evidence that TNF is produced by PMN in response to *C. albicans*. Culture of PMN with *C. albicans* for up to 8 hours was required for optimal induction of TNF. The suggestion of the need for new protein synthesis by the slow kinetics of TNF induction was confirmed using inhibitors of RNA and protein synthesis. Not only did cycloheximide and emetine inhibit the production and release of TNF, so did actinomycin D, which inhibits RNA synthesis. Thus, interaction of PMN with *C. albicans* induces a cycle of messenger RNA transcription and translation, leading to TNF release. The TNF class of molecule was verified by neutralization with specific MoAbs against TNF.

In addition to *C. albicans*, PMN could also be induced by bacterial LPS to release TNF. Therefore, the capacity for TNF production by PMN suggests an autocrine system for self-activation to muster host defense against microbes. However, TNF is also a potent activator of other cell

### Table 1. Neutralization TNF in PMN Supernatant Induced by *C. albicans*

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<th>Sample</th>
<th>Antibody</th>
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functions. For example, it can activate fibroblast cell growth, act as a chemotactic factor for monocytes, activate eosinophils against parasites, activate macrophages against intracellular bacteria, as well as up-regulate interleukin-2 receptors on NK cells. PMN are responsible for a rapid acute response to invading organisms, and the discovery of TNF release by PMN suggests that PMN may play a larger role in immune responses than previously realized. It is possible that PMN are not passive end effector cells responding to cytokines released by other cells, but may themselves actively participate in efficient generation of the immune response against microorganisms via cytokine release.

REFERENCES

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