Production of Granulocyte-Macrophage Colony-Stimulating Factor by Large Granular Lymphocytes Stimulated With Candida Albicans: Role in Activation of Human Neutrophil Function

By D. Kay Blanchard, M. Beatriz Michelini-Norris, and Julie Y. Djeu

In the present study, culture supernatants from larger granular lymphocytes (LGL) that were activated with Candida albicans antigens were shown to stimulate the ability of neutrophils to inhibit fungal growth. Identification of the activation factors showed that granulocyte-macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor, was involved. Human peripheral blood mononuclear cells were fractionated by Percoll density centrifugation and each subpopulation of cells was stimulated with C albicans yeast cells. GM-CSF was produced in those fractions enriched for LGL, but not T lymphocytes or adherent monocytes. Additionally, the phenotype of the GM-CSF-producing cell was found to be CD2+, CD16+, HLA-DR+, and negative for CD4, CD8, and CD15. Kinetic studies demonstrated that GM-CSF appeared in the supernatants within 2 days of culture and continued to be produced up to 7 days. Optimal stimulation of LGL was seen at a ratio of 3 heat-killed C albicans yeast cells per LGL. Thus, LGL play an important role in host defense against this opportunistic pathogen by producing cytokines, including GM-CSF, which in turn activates the fungicidal activity of neutrophils.

"COLONY-STIMULATING FACTORS (CSFs) are glyco-proteins that are characterized by their ability to induce the proliferation and differentiation of hematopoietic progenitor cells. It has also become evident that these CSF can enhance the effector functions of mature myeloid lineage cells. For example, human monocyte cytotoxicity can be activated by granulocyte-macrophage CSF (GM-CSF) and interleukin-3 (IL-3), apparently via induction of tumor necrosis factor (TNF) release. With regard to other cytokines, GM-CSF augments IL-1 production and can indirectly boost release of IL-2 by stimulating antigen-processing cells. CSFs are also active in enhancing antimicrobial activities of phagocytes. GM-CSF and macrophage CSF (M-CSF) have been shown in separate reports to enhance murine macrophage function against Leishmania tropica and Candida albicans. Our recent studies have also demonstrated a potent role for GM-CSF in the activation of human monocytes to kill C albicans. Incubation with as little as 0.1 U/mL was able to enhance their fungicidal activity against this opportunistic pathogen. GM-CSF also affects the functional activity of granulocytes by inhibiting migration, stimulating phagocytosis of Staphylococcus aureus, augmenting oxidative metabolism, and enhancing the in vitro survival of human polymorphonuclear leukocytes (PMN) and eosinophils.

Typically, GM-CSF is a product of activated T lymphocytes, although recent reports by Pistola et al19 and Cuturi et al20 have described the release of hematopoietic growth factors by natural killer (NK) cells. NK cells, or large granular lymphocytes (LGL), are known to produce a variety of cytokines, including TNF, interferon-γ (IFN-γ), IL-2, and IL-1. In an earlier report, we described the release of PMN-activating factors by Candida-stimulated LGL, and identified the presence of both TNF and IFN-γ. However, antibodies to these cytokines could not completely neutralize the PMN-activating activity in LGL supernatants, indicating that another factor was also involved.

C albicans is an opportunistic pathogen that colonizes man at birth and remains commensal in the gastrointestinal tract, skin, and mouth unless the host becomes immunocompromised. Secondary candidal infections frequently occur in patients undergoing immunosuppressive therapy for organ transplantation or for chemotherapy. In addition, those patients with genetic disorders linked to immunodeficiency or with acquired immunodeficiency syndrome (AIDS) often succumb to persistent candidal infections. These observations underline the importance of an intact immune system for resistance to this fungus.

While cell-mediated immune mechanisms play an important role in resistance to Candida, the final effector cell appears to be the phagocyte, either the PMN or the monocyte. Using Percoll density gradient fractionation, negative selection by specific antibodies plus complement, and positive selection by panning, LGL were identified as the source of PMN-activating factors, of which GM-CSF played a central role as a mediator between LGL and PMN.

MATERIALS AND METHODS

Culture of C albicans. C albicans used in this study was a clinical isolate from a patient with chronic mucocutaneous candidiasis. The yeast was grown by weekly transfer onto fresh Sabouraud’s agar plates and incubated at 25°C. Yeast cells obtained from agar plates were washed in phosphate-buffered saline (PBS; M.A. Biologics, Walkersville, MD) and were killed by heating in a boiling water bath for 1 hour.

Preparation of human leukocytes. Leukocyte buffy coats, obtained from normal volunteers at the Southwest Florida Blood Bank, were diluted 1:2 in PBS and centrifuged over Ficoll-Hypaque solution (Pharmacia, Piscataway, N.J). The peripheral blood mononuclear cells (PBMC) at the interphase were collected and washed twice with PBS and were suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 5% heat-inactivated human AB serum (Flow Laboratories, McLean, VA).

From the Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, FL.


Supported by National Institute of Allergy and Infectious Diseases Grant No. AI-24699.

Address reprint requests to D. Kay Blanchard, PhD, Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd, Tampa, FL 33612.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

mmol/L L-glutamine, 10 U/mL penicillin, 100 μg/mL streptomycin, 5 mmol/L HEPES buffer (GIBCO), and 5 × 10⁻³ mol/L 2-mercaptoethanol (Sigma Chemical Co, St Louis, MO), and will subsequently be referred to as complete medium. Plasticware was purchased from COSTAR (Cambridge, MA). Recombinant human GM-CSF (specific activity of 4 × 10⁶ colony-forming units [CFU]) per mg protein) was a very generous gift from Dr Steven C. Clark (Genetics Institute, Cambridge, MA). All media contained less than 0.1 ng/mL of endotoxin as determined by the Limulus Lysate assay (M.A. Biologies).

Preparation of monocytes. PBMC were incubated on gelatin-coated tissue culture flasks for 1 hour at 37°C to allow adherence of monocytes. Nonadherent cells (NAC) were recovered by vigorous washing of the flasks with warm medium and the adherent cells were removed by vigorous pipetting after the addition of cold PBS. Cells were then washed and resuspended in complete medium.

Discontinuous Percoll density gradient centrifugation. The separation of LGL from T cells was accomplished by the use of a discontinuous Percoll density gradient. NAC were further depleted of adherent cells and B cells by incubation on nylon wool columns for 30 minutes at 37°C. The cells passing through the columns were then placed on a six-step discontinuous density gradient with a range from 40% to 52.5% Percoll, as previously described. After centrifugation at 550g for 30 minutes at room temperature, the bands of lymphocytes were collected and examined for LGL morphology on Giemsa-stained cytocollofuged slides. In this series of experiments, fractions 2 and 3 contained 70% to 80% LGL and represented 10% to 15% of total peripheral blood lymphocytes (PB).

Serologic depletion studies. To determine the phenotype of the cells responsible for GM-CSF production, 4 × 10⁵ washed LGL per treatment were pelleted in a 5-mL plastic tube. A previously determined optimal dilution of each monoclonal antibody (MoAb) was then added to the cell pellet and incubated for 20 minutes at 37°C. The MoAbs recognizing CD4, CD8, and CD11 were obtained from Ortho Pharmaceutical Corp (Raritan, NJ), and antibodies to CD15, CD16, and HLA-DR were obtained from Becton Dickinson Co (Mountain View, CA). Low-toxic rabbit complement (Cedarlane, Westbury, NY) was then added to the antibody-treated cells at a 1:10 dilution and incubated from an additional 45 minutes to effect lysis. Cells were examined for viability by trypan blue exclusion, washed twice in RPMI medium, and resuspended to the volume corresponding to the cell concentration of untreated LGL (2 × 10⁶ cells/mL) without adjusting for nonviable cells to avoid enrichment of interfering cell populations in the experiments.

Positive selection by panning. To obtain a purified population of LGL, CD16+ cells were isolated by panning, as described by Engleman et al. For this procedure, the 3G8 clone (anti-CD16, IgG1) was used and was generously provided by Dr J. Unkeless (Mount Sinai Medical School, New York, NY). Briefly, approximately 4 × 10⁶ LGL were incubated with 100 μg of purified anti-CD16 antibody (MoAb) for 20 minutes at room temperature. Unbound antibody was removed by washing twice with 5% fetal calf serum (FCS) in PBS. Cells were then resuspended in 5 mL of 5% FCS in PBS and incubated for 2 hours at 4°C on a large tissue culture flask previously coated with goat antinmouse IgG (F(ab')₂ fragments; Sigma). Nonadherent cells (CD16-negative) were removed by four gentle washes with 1% FCS in PBS, and adherent cells (CD16-positive) were collected by vigorous pipetting with PBS. Cells were then washed with PBS and resuspended in medium for assay, as described. For these assays, 20% to 30% of CD16-coated cells from Percoll fractions 2 and 3 were nonadherent.

Induction of GM-CSF. Lymphocytes were cultured in 24-well tissue culture dishes at a concentration of 5 × 10⁹ cells/mL with 1 mL per well. Heat-killed candida yeast cells were added at the indicated ratios and the cocultures were incubated for 1 to 7 days at 37°C. Supernatant fluids were collected and passed through a 0.22-μm filter (COSTAR) to eliminate cell debris. Samples were either assayed immediately or were stored at −70°C.

Assay for GM-CSF. The presence of CSF in supernatants was assayed using the human Mo7e cell line, which was established from the peripheral blood of a patient with acute megakaryocytic leukemia. This cell line was a generous gift from Dr Steven C. Clark (Genetics Institute) and is maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 20% FCS (HyClone Labs, Logan, UT), glutamine, penicillin, and streptomycin. Recombinant human IL-3 or GM-CSF is a required growth factor and is added at a final concentration of 8 U/mL for continuous culture. For assay, Mo7e cells were washed twice with PBS, resuspended at a concentration of 1 × 10⁶ cells/mL in DMEM without growth factors, and 0.1 mL of the cell suspension was added to each well of a 96-well U-bottomed microtiter plate in which supernatant fluids were serially diluted in a final volume of 0.1 mL. Triplicate cultures for each dilution were incubated for 48 hours at 37°C and then pulsed with 0.5 μCi [methyl-³H]-thymidine (specific activity, 6.7 Ci/mmol; ICN Pharmaceuticals, Irvine, CA) per well. The cultures were incubated an additional 18 hours, harvested, and counted in a beta scintillation counter. A standard curve using recombinant human GM-CSF was included with each assay and was used to calculate units of CSF activity in supernatant fluids.

To characterize the growth factor produced by LGL, supernatants that supported the growth of Mo7e cells were neutralized with sheep anti-recombinant human GM-CSF antibodies or rabbit anti-recombinant human IL-3 antibodies. All antibodies were generously provided by Genetics Institute. An excess amount of antibody was added to an aliquot of supernatant fluid, incubated at ambient temperature for 30 minutes, and then assayed for residual growth factor activity.

NK assay. The NK assay was performed as described previously. Briefly, K562 erythroleukemia cells were labelled with sodium [³⁵Cr] chromate (Amersham Corp, Arlington Heights, IL), washed twice in PBS, and resuspended in complete medium at a concentration of 5 × 10⁶ cells/mL. K562 cells were then added to 96-well microtiter plates at 0.1 mL/well and used as target cells in a 4-hour ³⁵Cr-release assay with LGL as effector cells in E/T ratios varying from 20:1 to 5:1. The spontaneous control was usually less than 15%, and the standard error was less than 5%. The percent NK activity was then calculated as follows:

\[
\text{Assay for growth inhibition of } C \text{ albicans. The optimal conditions for assessment of } C \text{ albicans growth inhibition were as previously described. Briefly, PMN were diluted to } 1 \times 10^3, 3 \times 10^3, \text{ and } 1 \times 10^4 \text{ cells/mL in medium containing } 1 \% \text{ FCS, and } 0.05 \text{ mL of each dilution was added to triplicate wells of a } 96\text{-well flat-bottomed microplate. Then, } 0.05 \text{ mL of } C \text{ albicans at } 1 \times 10^4 \text{ yeast cells/mL in } 1 \% \text{ FCS-medium were added to all wells containing PMN, yielding E/T ratios of } 100:1, 30:1, \text{ and } 10:1. \text{ Candida were also added to empty wells to serve as controls. Cell cocultures were incubated for 18 hours at } 37°C \text{ and then pulsed with } 0.05 \text{ mL of } [\text{H}]\text{glucose (NET 807, D-[5,6-³H]glucose; New England Nuclear, Boston, MA)} \text{ diluted to } 10 \mu \text{Ci/mL in sterile water for an additional 3 hours of incubation. Candidal growth was collected with a Skatron harvester after the addition of } 0.05 \text{ mL of } 5.25 \% \text{ sodium hypochlorite to all wells. The mean of triplicate cultures was determined, and the standard error was within } 5\% \text{ of the mean. The percent growth inhibition (}\%\GI\text{ of } C \text{ albicans was calculated as follows: } \%\GI = (1 - [cpm of PMN and Candida/cpm Candida alone]) \times 100.}
GM-CSF FROM LGL STIMULATES PMN TO KILL FUNGI

were used as a control.

produce both TNF and IFN-γ, and that both of these cytokines are capable of augmenting the fungicidal activity of PMN.* However, neutralization studies using anti-TNF and anti-IFN-γ were unable to completely abrogate the stimulatory effect of LGL supernatants, leading to the speculation that CSF may be the unidentified PMN-activating factor. For this study, supernatants were generated by stimulating LGL with heat-killed yeast particles at a ratio of 10 yeasts per LGL for 30 minutes before the addition of Candida. Data are representative of three experiments performed with similar results.

The indicated supernatants were added to the PMN in each well at a final dilution of 1:100. The PMN and Candida were incubated for 18 hours at 37°C. The inhibition of fungal growth after incubation was calculated as a percentage of fungal growth inhibition (GI).

To confirm the stimulatory effect of GM-CSF on PMN, recombinant human GM-CSF was added to PMN 30 minutes before the addition of Candida in a growth inhibition assay. The inhibition of fungal growth after 18 hours of incubation is shown at the given E/T ratios of PMN to Candida.

Table 1. Induction of PMN-Activating Factor From LGL: Neutralization With Anti–GM-CSF

<table>
<thead>
<tr>
<th>Stimulant†</th>
<th>Candida Alone</th>
<th>30/1</th>
<th>10/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33,220 ± 1,375</td>
<td>30,015 ± 1,512 (9.7)</td>
<td>30,117 ± 1,478 (9.3)</td>
</tr>
<tr>
<td>LGL‡</td>
<td>32,939 ± 1,267</td>
<td>30,269 ± 1,743 (6.1)</td>
<td>31,515 ± 1,160 (4.3)</td>
</tr>
<tr>
<td>LGL + Ca†</td>
<td>33,881 ± 1,074</td>
<td>20,307 ± 984 (40.1)</td>
<td>23,579 ± 1,022 (30.4)</td>
</tr>
<tr>
<td>+anti-GM-CSF</td>
<td>33,208 ± 1,640</td>
<td>30,816 ± 1,511 (7.2)</td>
<td>29,177 ± 1,333 (12.1)</td>
</tr>
<tr>
<td>+anti–IL-3</td>
<td>35,675 ± 1,698</td>
<td>18,837 ± 886 (42.4)</td>
<td>23,833 ± 1,056 (27.1)</td>
</tr>
</tbody>
</table>

*PMN and Candida were incubated for 18 hours at 37°C at the indicated E/T ratios before [3H]glucose was added for an additional 3 hours for radiolabel uptake into residual Candida. Data are representative of three experiments performed with similar results.

†The indicated supernatants were added to the PMN in each well at a final 1:100 dilution 30 minutes before Candida was added.

‡Supernatants were generated by incubating LGL in medium alone or in the presence of Candida and the conditions of optimal stimulation by GM-CSF in stimulated LGL supernatants by negative selection, using specific antibodies that neutralize PMN-activating capacity of the supernatants. In the next series of experiments, the induction of GM-CSF from LGL was explored by the detection of known biologic function, ie, the ability to induce proliferation of a CSF-dependent hematopoietic cell line. The phenotype of the activated LGL and the conditions of optimal stimulation by Candida were characterized by using the Mo7e cell line to assess the biologic activity of supernatants from activated LGL under various conditions.

**Dependence of Mo7eproliferation on CSF.** Before assessment of CSF activity with the Mo7e cell line, it was first necessary to establish their experimental sensitivity only to CSF, and not other unrelated cytokines. For this experiment, a variety of recombinant cytokines was serially diluted and added to Mo7e cells before measurement of cell proliferation (Table 3). Of the hematopoietic growth factors used, only GM-CSF and IL-3 were found to support the growth of Mo7e. G-CSF and M-CSF, which are reportedly required for differentiation of progenitor cells at a relatively late stage of their development, were unable to maintain proliferation of this leukemic cell line. Because TNF is reportedly a growth factor for some cells, such as donor PMN to inhibit the growth of Candida differed, the PMN of both donors were activated by the addition of as little as 0.1 U/mL of GM-CSF. Thus, PMN fungicidal activity can be enhanced by very small amounts of this cytokine.

Table 2. Effect of Recombinant GM-CSF on Growth Inhibition of Candida by PMN

<table>
<thead>
<tr>
<th>Donor</th>
<th>GM-CSF (U/mL)</th>
<th>100/1</th>
<th>30/1</th>
<th>10/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>64.0</td>
<td>33.0</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>75.1</td>
<td>67.9</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>67.9</td>
<td>67.4</td>
<td>51.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>76.7</td>
<td>59.8</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>66.3</td>
<td>65.8</td>
<td>37.9</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>67.5</td>
<td>31.3</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>93.9</td>
<td>95.9</td>
<td>75.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>93.5</td>
<td>90.7</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>91.9</td>
<td>87.8</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>90.9</td>
<td>85.0</td>
<td>45.5</td>
</tr>
</tbody>
</table>

PMN from each donor were incubated with the indicated concentration of recombinant human GM-CSF for 30 minutes before the addition of Candida. The inhibition of fungal growth after 18 hours of incubation is shown at the given E/T ratios of PMN to Candida.
fibroblasts, it was also assessed for this function with Mo7e. As shown, neither TNF nor other monokines, eg, fibrinblasts, it was also assessed for this function with 3H-thymidine. The cpm of Mo7e in medium alone was 2262 BLANCHARD, MICHELINI-NORRIS, AND DJEU proliferation of Mo7e induced by 10 U/mL of GM-CSF, would not affect the detection of CSF by Mo7e cells. Alternatively, it was possible that TNF might be cytotoxic for these leukemic cells. Because we have shown that TNF can also be produced by Candida-stimulated LGL, it was important to determine whether this factor might mask the detection of CSF in the present assay. Table 3 shows that 1,000 U/mL of TNF had no effect on the proliferation of Mo7e induced by 10 U/mL of GM-CSF, indicating that the presence of TNF in LGL supernatants would not affect the detection of CSF by Mo7e cells.

Induction of CSF by C albicans and the identification of the CSF-producing cell population. To define which cells from normal peripheral blood responded to fungal antigens by producing hematopoietic growth factors, various subpopulations of PBL were isolated by adherence, to yield monocytes, and by Percoll fractionation, to separate LGL from T lymphocytes. The cell subpopulations were then similarly stimulated with C albicans for 3 days and their supernatants were collected and assessed for CSF content. As shown in Fig 1, PBL were found to respond to stimulation by CSF production. Fractionation of PBL into various subsets indicated that monocytes did not produce GM-CSF or IL-3 under such stimulation. CSF was also not found in the supernatants of T cells, which are isolated from Percoll fractions 4 through 7. However, cells from Percoll fractions 2 and 3, which contain the LGL subpopulation of lymphocytes, produced 42 and 67 U/mL of CSF activity, respectively, after 3 days of stimulation with C albicans yeast cells.

Kinetics of CSF production. The above experiments showed that C albicans was capable of inducing CSF from LGL within 3 days of culture. To determine the time course of growth factor production, LGL and monocytes were cocultured with C albicans for 1 to 7 days before the supernatants were assayed for CSF. As shown in Fig 2, little CSF activity was seen in the supernatants of C albicans-stimulated LGL until day 2 of culture. From that time, CSF continually increased throughout the 7 days of incubation. No CSF was seen in culture fluids of unstimulated LGL or unstimulated monocytes. Furthermore, only minimal levels of CSF (4 U/mL) were produced by stimulated monocytes compared with induced LGL (51 U/mL) after 7 days of incubation.

### Table 3. Effect of Various Cytokines on Proliferation of the Mo7e Cell Line

<table>
<thead>
<tr>
<th>Cytokine*</th>
<th>1,000 U/mL</th>
<th>100 U/mL</th>
<th>10 U/mL</th>
<th>1 U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>25,371 ± 922</td>
<td>6,709 ± 97</td>
<td>626 ± 24</td>
<td>108 ± 11</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>28,255 ± 870</td>
<td>18,546 ± 328</td>
<td>1,650 ± 69</td>
<td>188 ± 12</td>
</tr>
<tr>
<td>G-CSF</td>
<td>109 ± 20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M-CSF</td>
<td>118 ± 20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1a</td>
<td>112 ± 7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1β</td>
<td>145 ± 29</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-6</td>
<td>132 ± 12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF</td>
<td>143 ± 14</td>
<td>ND</td>
<td>ND</td>
<td>1,811 ± 77</td>
</tr>
<tr>
<td>TNF + GM-CSF†</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*The indicated cytokines were added to 10⁴ Mo7e cells in a final volume of 0.2 mL for 48 hours at 37°C, followed by an 18-hour pulse with 3H-thymidine. The cpm of Mo7e in medium alone was 127 ± 23.

†TNF at 1,000 U/mL was added to GM-CSF at 10 U/mL.

**Dose dependence of CSF production.** To determine whether a dose response to the number of stimulating C albicans yeast cells exists, LGL and monocytes were cultured with varying ratios of yeast to leukocyte, ranging from 0.3:1 to 30:1 (Fig 3). As few as 1 yeast cell per 3 LGL was found to induce detectable levels of CSF. Optimal stimulation of LGL was found at a ratio of 3 C albicans per lymphocyte, resulting in 53 U/mL after 3 days of incubation. As C albicans concentrations increase to 30:1, less CSF is produced, with levels decreasing to 19 U/mL. Alternatively, monocytes were not found to produce significant levels of CSF at any concentration of C albicans.

Neutralization of CSF with antibodies. Because Mo7e cells are shown to respond to both GM-CSF and IL-3, it was important to differentiate the type of CSF that was produced by Candida-stimulated LGL. For this experiment, supernatants that contained CSF activity were incubated with excess amounts of neutralizing antibody preparations and then assayed for residual growth factor activity. As shown in Table 4, treatment of LGL supernatants with...
sheep antirecombinant human GM-CSF neutralized 97.8% of CSF activity, indicating that C. albicans induced GM-CSF from LGL. On the other hand, rabbit antirecombinant human IL-3 minimally affected CSF activity in the LGL supernatants. The small decrease in activity by anti–IL-3 may be attributed to some crossreaction of this preparation with GM-CSF. This result can be seen by its ability to neutralize the proliferative activity of recombinant human GM-CSF by 15.4%. Due to the almost complete neutralization of CSF activity by anti–GM-CSF, the growth factor produced by stimulated LGL was identified as GM-CSF.

**Phenotype of CSF-producing LGL.** To determine the phenotype of the lymphocyte responsible for CSF production, LGL were serologically depleted of various subpopulations by incubation with MoAbs and complement to lyse the indicated phenotype. After complement depletion, the remaining LGL were stimulated with Candida for 3 days at a ratio of 3:1. An aliquot of LGL was also assessed for NK activity following complement depletion to determine if the CSF-producing LGL is associated with the subset known to display tumoricidal activity.

Treatment of LGL with anti-CD15 MoAbs, which depletes monocytes and macrophages, had only a slight effect on the production of CSF or on NK activity (Table 5). Further treatment with anti-CD4 and anti-CD8, which lyse T-helper and T-suppressor/cytotoxic lymphocyte subsets, respectively, did not affect CSF production, although NK activity was moderately decreased. Depletion of CD2-positive cells, which include T lymphocytes and LGL, abrogated both CSF production and NK activity. However, elimination of HLA-DR–positive LGL resulted in a loss of CSF activity, but NK-mediated lysis of K562 cells was not significantly affected. Finally, treatment of LGL with anti-CD16, which identifies NK cells, eliminated both CSF production and NK activity.

Because the use of complement plus anti-CD16 (Leu 11b) antibodies did not efficiently eliminate LGL in the Percoll-enriched populations, a positive selection technique was also used to identify the cell population responsive to Candida. Cells were coated with anti-CD16 (3G8 clone) and then separated into CD16-positive and -negative phenotypes by panning on flasks coated with goat anti-mouse IgG. As shown in Table 6, CD16-negative cells possessed neither NK activity nor the ability to produce CSF on stimulation with Candida, while both activities were seen within the CD16-positive population. Interestingly, treatment of LGL with anti-CD16 antibodies alone resulted in production of CSF, although in lower amounts than in the presence of Candida antigens. These results are similar to those of Cuturi et al., who reported that anti-CD16 (3G8) antibodies bound to Sepharose could

<p>| Table 4. Neutralization of CSF Activity from Candida-Stimulated LGL With Specific Antisera |
|---------------------------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Sample</th>
<th>Medium (cpm)</th>
<th>Anti-GM-CSF (cpm)</th>
<th>Anti-IL-3 (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>226 ± 31</td>
<td>198 ± 3</td>
<td>165 ± 16</td>
</tr>
<tr>
<td>rGM-CSF (30 U/mL)</td>
<td>5.265 ± 159</td>
<td>3.696 ± 8 (96.6)</td>
<td>4.414 ± 55 (15.4)</td>
</tr>
<tr>
<td>rIL-3 (30 U/mL)</td>
<td>4.036 ± 140</td>
<td>3.453 ± 240 (14.6)</td>
<td>197 ± 16 (90.2)</td>
</tr>
<tr>
<td>LGL + Ca</td>
<td>3.309 ± 108</td>
<td>266 ± 10 (97.8)</td>
<td>2.584 ± 57 (21.6)</td>
</tr>
</tbody>
</table>

Samples were incubated at ambient temperature for 30 minutes with an excess amount of the indicated antibodies and then assessed for residual CSF activity as detected by proliferation of Mo7e cell line. Supernatants were collected from LGL stimulated with C. albicans (Ca) yeast cells at a ratio of 3 yeasts per LGL for 2 days. Data are representative of three experiments that were performed with similar results.
induce LGL to produce GM-CSF. Thus, from the positive and negative selection methods, the phenotype of the LGL that responded to stimulation by C. albicans was CD2+, CD16+, and HLA-DR+.

**DISCUSSION**

PMN have been shown to be an important factor in resistance to opportunistic infection by C. albicans. Furthermore, their activity is sensitive to stimulation by a variety of cytokines, including IFN-γ and TNF, and, as demonstrated in this study, GM-CSF. As little as 0.1 U/mL of recombinant GM-CSF was found to significantly enhance the ability of PMN to inhibit fungal growth. The purpose of this study was to understand the role of LGL, a potent source of such cytokines, in resistance to opportunistic infections.

In the present study, LGL, but not monocytes or small, mature T cells, were found to produce GM-CSF when directly stimulated with C. albicans. As few as 1 yeast per 3 LGL could elicit sufficient levels of this cytokine to activate PMN fungicidal activity. Neutralization of CSF activity by anti-GM-CSF demonstrated that this factor was produced. While anti-IL-3 antibodies were found to decrease LGL CD16+, and HLA-DR+.

Table 5. Phenotype of CSF-Producing LGL by Serum Depletion

<table>
<thead>
<tr>
<th>Treatment of LGL (%) viability</th>
<th>GM-CSF (U/mL)</th>
<th>NK Activity at E/T Ratio (% lysis ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Ca</td>
</tr>
<tr>
<td>Complement only (99) &lt;1</td>
<td>40 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Anti-CD15 (94) &lt;1</td>
<td>31 ± 2</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>Anti-CD15/CD4/CD8 (51) &lt;1</td>
<td>32 ± 1</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Anti-CD15/CD2 (24) &lt;1</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Anti-CD15/HLA-DR (96) &lt;1</td>
<td>2 ± 1</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>Anti-CD15/CD16 (73) &lt;1</td>
<td>&lt;1</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

LGL were treated with the indicated antibodies plus complement and then incubated in medium alone or with C. albicans (Ca) at a ratio of 3:1 for 3 days. NK assays were performed at the indicated E/T ratios of effector to K562 tumor target cell in a 4-hour 51Cr-release assay. Data are representative of three experiments that were performed with similar results.

Table 6. Phenotype of CSF-Producing LGL by Panning

<table>
<thead>
<tr>
<th>LGL Population</th>
<th>GM-CSF (U/mL)</th>
<th>NK Activity at E/T Ratio (% lysis ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll 2/3 &lt;1</td>
<td>22 ± 3</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>CD16-negative &lt;1</td>
<td>&lt;1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>CD16-positive 7 ± 1</td>
<td>34 ± 4</td>
<td>30 ± 3</td>
</tr>
</tbody>
</table>

Cells from Percoll fractions 2 and 3 were separated by panning into CD16-negative and -positive populations and then incubated in medium alone or with C. albicans (Ca) at a ratio of 3:1 for 3 days. NK assays were performed at the indicated E/T ratios of effector to K562 tumor target cell in a 4-hour 51Cr-release assay. Data are representative of two experiments that were performed with similar results.
induced by C. albicans from LGL coupled with its ability to mobilize neutrophil or monocyte function, suggest that it may be a key cytokine that plays an important role in keeping this normally nonpathogenic organism under the control of LGL and phagocytes.

REFERENCES


ACKNOWLEDGMENT

We thank Dr Steven C. Clark (Genetics Institute) for his generous supply of GM-CSF and IL-3, and for the Mo7e cell line. We also thank Debbie Serbousek and Ann Taylor for their technical assistance.
Production of granulocyte-macrophage colony-stimulating factor by large granular lymphocytes stimulated with Candida albicans: role in activation of human neutrophil function

DK Blanchard, MB Michelini-Norris and JY Djeu