Inducible Production of Interleukin-6 by Human Polymorphonuclear Neutrophils: Role of Granulocyte-Macrophage Colony-Stimulating Factor and Tumor Necrosis Factor-alpha

By Nicola A. Cicco, Albrecht Lindemann, Jean Content, Paul Vandenbussche, Michael Lübbert, Jutta Gauss, Roland Mertelsmann, and Friedhelm Herrmann

The recent demonstration of the ability of human polymorphonuclear neutrophils (PMN) to secrete various cytokines in response to the granulocyte activator granulocyte-macrophage colony-stimulating factor (GM-CSF) but not to other cytokines, has led to the identification of PMN as biosynthetically active cells. In this study, we have investigated the ability of PMN to secrete interleukin-6 (IL-6), a molecule known to be involved in inflammatory reactions. Using RNA blotting analysis and bioassays, we show that PMN can be induced to synthesize transcripts specific for IL-6, indistinguishable in size from IL-6 mRNA produced by activated human macrophages. Consequently, PMN released IL-6-like activity into their culture supernatants that could be neutralized by monospecific anti-IL-6 antibody.

PMN were prepared from heparinized peripheral blood obtained from consenting healthy volunteers by density separation using Ficoll-Hypaque (Pharmacia Laboratories, Uppsala, Sweden), followed by dextran sedimentation and immune rosetting with monoclonal antibody 1D3, as described. The monoclonal antibody 1D3 reacting with neutrophils only was kindly provided by J. Griffin, Dana-Farber Cancer Institute, Boston, MA.

The resulting cell preparation contained more than 98% PMN by morphology, with only a few contaminating eosinophils. Culture conditions. Standard culture medium consisted of RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin, 1% sodium pyruvate, and 5% low endotoxin fetal calf serum (FCS). Neutrophil preparations were washed and resuspended at 5 × 10^6 cells/mL in standard culture medium in 24-well flat-bottomed plates (Corning, New York, NY), in the presence or absence of 50 ng/mL recombinant human (rh) GM-CSF, 50 ng/mL rhIL-3 (kindly provided by F.R. Seiler, Behringwerke, Marburg, FRG), 50 ng/mL rhG-CSF (kindly

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provided by L. Souza, Amgen, Thousand Oaks, CA), 1,000 U/mL rhM-CSF (kindly provided by P. Ralph, Cetus Corporation, Emeryville, CA), 50 ng/mL rhIFN-γ (kindly provided by S. Papendick, Thomaef GmbH, Biberach, FRG), 100 ng/mL rhLT (kindly provided by D. Blohm, BASF, Ludwigshafen, FRG), or 50 ng/mL rhTNF-α (kindly provided by G. Adolf, Boehringer, Vienna, Austria). In selected experiments, PMN were also exposed to phorbol ester phorbol myristate acetate (PMA; 5 x 10⁻⁴ mol/L), to the inhibitor of protein synthesis cycloheximide (CHX; 20 μg/mL), to Escherichia coli-derived lipopolysaccharide (LPS; 10 μg/mL), and to FMLP (5 x 10⁻⁴ mol/L) (Sigma; Munich, FRG). After 24 hours, cell-free supernatants of PMN cultures (PMN-CM) were collected and stored at -70°C until assayed. For RNA analysis, PMN were incubated at 2.5 x 10⁶/mL in standard culture medium under identical culture conditions as above.

Biologic assay for IL-6. Biologic activity of IL-6 was measured by its proliferative action on the IL-6-dependent hybridoma cell line B13.29 (subline B9). Proliferation was measured by means of a calorimetric assay. To this end, B9 cells were cultured at 10⁴ cells/mL in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 50 μmol/L 2-mercaptoethanol, 5% FCS, penicillin, and streptomycin for 78 hours in flat-bottomed microtiter plates (Corning). Six hours before harvesting, all cultures received 50 μL per well of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma). After another 4-hour incubation period, the supernatant was removed, and blue formazan crystals were dissolved in 0.01 N HCl/ethanol. The absorbance at 570 nm was measured by a multiwell scanning spectrophotometer (Titertek Multiscan, Flow Laboratories, McLean, VA). The results are expressed as U/mL (means ± SEM) of triplicate cultures. Recombinant IL-6 was used as an internal standard; 1 U/mL is defined as the concentration of IL-6 leading to half-maximal stimulation of B9 cells and equals 1 pg/mL IL-6. B9 cells do not proliferate in response to any of the inducing cytokines investigated. In selected experiments, supernatants of GM-CSF and TNF-α-induced PMN cultures were treated with a rabbit polyclonal antiserum directed against rhIL-6 (10³ neutralizing U/mL) for 2 hours at room temperature, before incorporation into the B9 proliferation assay (antiserum produced by P.V.).

Isolation of RNA. RNA blotting, and hybridization. Total cellular RNA was prepared using the guanidinium thiocyanate method. Polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography, according to the protocol described by Aviv and Leder. RNA electrophoresis was carried out under denaturing conditions in the presence of formamide. Gels were transferred to nitrocellulose (Schleicher and Schuell, Dassel, FRG).

IL-6 mRNA was detected by using a full length cDNA (pBSF2-38.1), as described. This probe was a kind gift of T. Hirano and T. Kishimoto, Division of Immunology, Osaka University, Osaka, Japan. To exclude the possibility that IL-6 was being produced by contaminating monocytes (Mo), PMN poly(A)⁺ RNA was re-probed with a v-fms specific cDNA (kindly provided by C.J. Sherr, St Jude Children’s Research Hospital, Memphis, TN) without demonstrating a specific hybridization signal (data not shown). As positive control for specific hybridization reactions, poly(A)⁺ RNA from Mo, activated with PMA (10⁻⁴ mol/L) was used. Blots that were done with either 3.5 μg PMN poly(A)⁺ RNA or 2.0 μg monocyte poly(A)⁺ RNA per lane were exposed to Kodak x-omat films with intensifying screens.

RESULTS

PMN and monocytes act as first line cells of host defence against infectious agents and play a pivotal role in acute inflammation. In addition, both cell types have several features in common, including their common cell lineage, phagocytic nature, similar receptors, and surface antigens, as well as the ability to produce soluble mediators such as IL-1, G-CSF, or M-CSF. In an attempt to study whether PMN could produce other inflammatory cytokines (particularly with regard to IL-6), the present study was initiated. We could not show a constitutive secretion of IL-6 by PMN, but after stimulation with recombinant human GM-CSF and TNF-α, PMN were capable of releasing IL-6, as demonstrated when cell-free supernatants of GM-CSF or TNF-α–treated PMN cultures (GM-CSF PMN-CM; TNF-α PMN-CM) were assayed for their capacity to induce proliferation of B9 cells that could be blocked by previous treatment of PMN-CM with neutralizing concentrations of a monospecific antibody to human IL-6. However, supernatants of PMN cultures stimulated with other cytokines did not contain detectable amounts of biologic IL-6 activity (Table 1).

IL-6 mRNA was also detected in human PMN. IL-6 specific transcripts accumulated early after the onset of the GM-CSF treatment of PMN cultures, with a maximum level reached at 6 hours (Fig 1), whereas no IL-6-specific mRNA was detected in the absence of GM-CSF (data not shown), or in the presence of culture of IL-3, G-CSF, M-CSF, IFN-γ, and LT (Fig 2).

<p>| Table 1. GM-CSF and TNF-α Induce Secretion of IL-6 by PMN |</p>
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL-6 (U/mL)</th>
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<tbody>
<tr>
<td>Medium</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
</tr>
<tr>
<td>G-CSF</td>
<td>ND</td>
</tr>
<tr>
<td>M-CSF</td>
<td>ND</td>
</tr>
<tr>
<td>IL-3</td>
<td>ND</td>
</tr>
<tr>
<td>LT</td>
<td>ND</td>
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<tr>
<td>TNF-α</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF PMN-CM</td>
<td>240 ± 17</td>
</tr>
<tr>
<td>G-CSF PMN-CM</td>
<td>ND</td>
</tr>
<tr>
<td>M-CSF PMN-CM</td>
<td>ND</td>
</tr>
<tr>
<td>IL-3 PMN-CM</td>
<td>ND</td>
</tr>
<tr>
<td>LT-PMN-CM</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α PMN-CM</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>IFN-γ PMN-CM</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF PMN-CM + anti-IL-6</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>TNF-α PMN-CM + anti-IL-6</td>
<td>ND</td>
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</table>

PMN (5 x 10⁶/mL) were cultured in the presence or absence of various recombinant human cytokines (see Materials and Methods for protocol). After 24 hours of culture, cell-free culture supernatants were collected and measured for IL-6 activity using the IL-6–dependent hybridoma line B13.29 (subline B9). B9 cells were cultured at 10⁶ cells per mL in medium supplemented with supernatants (10% vol/vol) of PMN cultures (PMN-CM) that were generated in the presence or absence of cytokines (as above), in medium only, or in medium that contained cytokines in the same concentrations that were used for induction of PMN. Values are expressed as means ± SEM of triplicate cultures. The detection level of IL-6 was 5 U/mL. Abbreviation: ND, not detectable.
DISCUSSION

The ability of PMN to secrete IL-6 after GM-CSF induction bears several implications. First, as outlined above, IL-6 was shown to synergistically promote growth inducing activity on the level of multipotential normal hematopoietic progenitor cells in concert with IL-1 and IL-3. PMN, therefore, may be involved in the proliferation of their own progenitors by establishing a positive feedback loop via release of IL-6. Moreover, the importance of the acute phase proteins (APP) in the innate immunity to infection is well known. Some of the APP can act as opsonins and activate the complement network.

Since IL-6 has been described as one of the major inducers for APP in hepatocytes, this study suggests that PMN, besides their "traditional" role as cells with phagocytic properties, may exert a key function as regulators of the humoral response during the evolution of an inflammatory reaction. As demonstrated from cell quantities needed to obtain comparable hybridizing signals, the specific capacity per cell is about 10 times less in PMN as compared with Mo.

REFERENCES

7. Weisbart RH, Kwan L, Golde DW, Gasson JC: Human granulocyte-macrophage colony-stimulating factor primes neutro-
phils for enhanced oxidative metabolism in response to the major physiological chemottractants. Blood 69:18, 1987


Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha

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