Effects of γ-Interferon on Human Neutrophils: Protection From Deterioration on Storage

By Seymour J. Klebanoff, Slawomir Olszowski, Wesley C. Van Voorhis, Jeffrey A. Ledbetter, Ann M. Waltersdorff, and Kristie G. Schlechte

Human polymorphonuclear leukocytes (PMN) preincubated overnight with 100 U/mL γ-interferon (IFN-γ) had an increased metabolic response, as measured by iodination and/or superoxide production, to stimulation by tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), formylmethionyl-leucyl-phenylalanine (FMLP), opsonized zymosan, and lipopolysaccharide (LPS), as compared with cells comparably preincubated in the absence of IFN-γ. The decline in the staphylocidal activity of the stored PMN was also prevented in part by IFN-γ, as was the depressed adherence of PMN stimulated with phorbol myristate acetate (PMA), FMLP, TNF, GM-CSF, and LPS. This protective effect of IFN-γ on PMN function was associated with the prolonged surface expression of the complement receptor three (CR3) α-chain (CD11b), CR5 β-chain (CD18), FeRRI (CD32), and FcRII (CD16), and the appearance of surface FcR (CD64). The polymerase chain reaction (PCR) was used to amplify neutrophil RNA-derived cDNA recognized by synthetic oligonucleotides designed from published nucleotide sequences for specific proteins. Using this procedure, mRNA for gp91-phox, p67-phox, p47-phox, CD64, two forms of CD32, CD16, CD11b, CD18, and actin were found to be depressed after overnight storage of neutrophils, and this decrease in steady-state mRNA levels was in part or totally prevented by IFN-γ. CD64 and gp91-phox mRNA were generally increased by IFN-γ to a level greater than that of freshly isolated neutrophils. Northern analysis of CD64 and p47 phox mRNAs confirmed the findings with the PCR method. These findings suggest that storage of PMN in a functionally active state is favored by the presence of IFN-γ.

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mol/L sodium chloride, 1.2 × 10⁻² mol/L potassium chloride, 10⁻³ mol/L calcium chloride, 2 × 10⁻³ mol/L magnesium chloride, and 2 × 10⁻³ mol/L glucose.

PMN. Venous blood was collected from normal volunteers just before cell separation using 0.2% dipotassium ethylenediaminetetraacetic acid (K₂EDTA) as anticoagulant. The blood was mixed with an equal volume of 3% Dextran T-500 (Pharmacia LKB Biotechnology, Piscataway, NJ) in 0.9% sodium chloride and the erythrocytes allowed to settle at room temperature for 20 to 30 minutes. The supernatant layer was centrifuged for 5 minutes at 350g at 10°C and the combined cell pellets were suspended in 30 ml of 0.9% sodium chloride. The cell suspension was underlaid with 15 ml of cold Histopaque-1077 (Sigma Chemical Co, St Louis; MO) and centrifuged for 20 minutes at 1,000g and 10°C. The supernatant was aspirated to approximately 0.5 cm above the PMN layer and the sides of the tube were cleaned with a dry cotton swab to remove contaminating adherent cells. For hypotonic lysis of contaminating erythrocytes, 10 to 20 ml ice-cold 0.2% sodium chloride was added to the cell pellet, the tube was vortexed for 25 seconds and the isotonicity immediately restored with an equal volume of 1.6% sodium chloride. A second hypotonic lysis was performed if the cell pellet remained red. The cell suspension was centrifuged for 5 minutes at 350g at 10°C and the cell pellet suspended in 0.9% sodium chloride. The preparation contained greater than 97% PMN, of which 5.1% were eosinophils, and is termed PMN. In some experiments in which mRNA levels were measured, the neutrophils were further purified by centrifugation over Metrizamide (Nyegaard & Co, Oslo, Norway) gradients. Total cell preparations contained less than 1% eosinophils and no detectable monocytes and are termed neutrophils. Cytocentrifuge smears of the cell preparations were routinely stained with Diff-Quick (American Scientific Products, McGaw Park, IL) for morphologic examination and trypan blue exclusion showed the viability to be greater than 96% for all preparations.

The cells were suspended in RPMI 1640-10% FCS at 5 × 10⁶ PMN/mL. Ten to 15 ml of the cell suspension was preincubated either alone, with 100 U/mL IFN-γ, or with 100 U/mL IFN-γ + 0.1 µL/mL polyclonal antihuman IFN-γ in 50 mL tissue culture flasks (no. 3055; Costar, Cambridge, MA) for 18 to 20 hours at 37°C in a humidified CO₂ incubator (5% CO₂–95% air). The cells were collected by centrifugation at 750g for 5 minutes at 4°C, washed twice in Hank’s balanced salt solution containing 1 mmol/L calcium chloride and 2 mmol/L magnesium chloride (HBSS), suspended in 0.9% sodium chloride, and used within 30 minutes. All the reagents used in the cell preparation and overnight culture were tested by the limulus amebocyte lysate assay (Sigma) for endotoxin, and found to contain less than 20 pg/mL.

Iodination. The conversion of radioiodide to a trichloroacetic acid (TCA)-precipitable form was used as a measure of iodination. The reaction of the component mixture, as described in the legends to the figures and tables, were added to 12 × 75 mm polystyrene test tubes (Falcon 2054; Becton Dickinson, Oxford, CA) in a final volume of 0.5 mL and maintained in a water bath without shaking for 60 minutes at 37°C. The reaction was stopped by the addition of 1.0 mL of cold 10% TCA and the precipitate collected, washed and counted, as previously described.30 The results are expressed as picomoles of iodide converted to a TCA-precipitable form per 10⁶ PMN per hour. Each experimental value was determined in duplicate, and the average was used as a single n for statistical analysis.

Superoxide anion (O₂⁻) production. Superoxide anion production by PMN was determined by superoxide dismutase-inhibitable ferricytochrome C reduction. The components of the reaction mixture (see legend to the table) were incubated in 12 × 75 mm polystyrene test tubes at 37°C for 60 minutes without shaking. Each reaction tube was run in parallel with an identical tube, except for the addition of 120 µg/mL superoxide dismutase. At the end of the incubation, the reaction mixture was centrifuged at 1,000g for 6 minutes at 4°C and placed on ice. The concentration of ferrocyanochrome C was determined spectrophotometrically at 550 nm using E = 2.1 × 10⁴ M⁻¹ cm⁻¹.

Bactericidal activity. Staphylococcus aureus (502A) was maintained on blood agar plates and, just before the experiment, transferred to Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) and grown at 37°C on a Fisher Roto-Rack (Fisher Scientific Co, Pittsburgh, PA) rotating 15 times per minute for 2 hours. The organisms were collected by centrifugation, washed twice with 0.1 mol/L sodium sulfate, and suspended in 0.1 mol/L sodium sulfate to the required absorbency at 540 nm. The S. aureus were incubated with the components indicated in the legend to Fig 2 in a final volume of 0.5 mL at 37°C with tumbler, and the viable cell count was determined by the pour-plate method using trypticase soy agar.32

Adherence. PMN (10 × 10⁶/mL) in HBSS containing 0.01% gelatin were incubated with 51Cr-chromate (100 µCi/mL) for 45 minutes at 37°C with shaking. The cells were washed twice, suspended in HBSS with 0.5% bovine serum albumin (Fraction V; Sigma), and 4 × 10⁵ cells in 100 µL were added per well to 96-well vinyl tissue culture plates (Costar 2596; Cambridge, MA). The plates were incubated at 37°C in a 5% CO₂ incubator for the periods indicated, the medium and nonadherent cells removed by gentle vacuum suction, and a stream of HBSS directed forcefully into the well using a 10-cm³ syringe with a 22-gauge needle. Washing was repeated four times, the vinyl wells cut from the plate, placed in a plastic tube, and counted in a gamma scintillation counter. A standard containing the total amount of 51Cr in the reaction mixture was counted and the percent adherence determined. Background adherence, ie, adherence of unstimulated PMN, was less than 10% under these conditions.

Immunofluorescence flow cytometry. PMN were incubated with or without 100 U/mL of IFN-γ for periods up to 48 hours, washed in HBSS, and suspended in 0.9% sodium chloride at a concentration of 2 × 10⁶ per mL, as described above. An aliquot of the PMN suspension was used to make a final concentration of 2 × 10⁶/mL was added to 12 × 75 mm polystyrene test tubes (Falcon 2054; Becton Dickinson, Lincoln Park, IL) containing the standard salt solution and 0.25 mg Cr solution in a final volume of 0.5 mL. The tubes were kept at room temperature for 5 minutes and then placed on ice for 5 minutes. An aliquot of the cell suspension (50 µL, 10⁶ PMN) was added to 50 µL of the MoAb in wells of a 96-well vinyl assay plate (Costar 2596; Cambridge, MA) which was kept on ice. The MoAb was dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% sodium azide (PBS-Alb-Az). The MoAbs used were: 60.1, an IgG1 antibody that binds to CD11b (CR3 α-chain)33; 60.3, an IgG2a antibody that binds to CD18 (CR3 β-chain)34; 32.2, an IgG1 antibody that binds to CD64 (FcRII)35; IV-3, an IgG2b antibody that binds to CD32 (FcRII)36; FC-2, an IgG2b antibody that binds to CD16 (FcRII)37. MoAbs 60.1, 60.3, IV-3, and FC-2 were directly conjugated with fluorescein isothiocyanate (FITC) as described,38 whereas MoAb 32.2 was unconjugated. The plates were incubated for 45 minutes at 4°C, and the cells incubated with FITC-conjugated MoAbs were washed once with PBS containing 0.1% sodium azide and fixed with 1% formaldehyde.39 PMNs incubated with MoAb 32.2 were washed once with PBS-Alb-Az, incubated with FITC-conjugated goat F(ab′)₂ antiserum, and were used with PBS containing 0.1% sodium azide and fixed with 1% formaldehyde. PMN incubated with MoAb 32.2 were washed once with PBS-Alb-Az, incubated with FITC-conjugated primary antibody. All MoAbs were present in saturating amounts. The plates were kept on ice until the PMN...
were analyzed by flow cytometry using a Coulter Epics C fluorescence-activated cell sorter.

**Oligonucleotide primer design.** Oligonucleotide primers for polymerase chain reaction (PCR) design of cDNA specific for the phagocyte NADPH oxidase components gp91-phox, p47-phox, p67-phox, for the Fc receptors FcRI (CD64), two forms of FcRII (CD32/PC and CD32/TC), and FcRIII (CD16) and for the CR3 subunits CD11b and CD18 were synthesized (Table 1). FcR primers were designed to anneal in the regions of lowest homology. As a control for specificity, interchangre of 5' and 3' primers between CD32/PC and CD32/TC, which have a high degree of homology, did not lead to any PCR products.

**Detection of mRNA by PCR.** Total RNA was isolated from 8 x 10^7 PMN using guanidium isothiocyanate lysis and cesium step gradient centrifugation. The average yield of RNA in different donors varied from 12 to 18 μg/10^6 cells. RNA was reverse transcribed into cDNA as follows. One microgram of denatured total RNA was added to a reaction mixture containing 50 mmol/L Tris-HCl buffer pH 8.3, 75 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 10 mmol/L dithiothreitol, 10 U RNAse (Promega, Madison, WI), 0.5 mmol/L dNTPs (dATP, dCTP, dGTP, and dTTP; Pharmacia), 0.025 mg/mL random hexadeoxynucleotide primers, and 200 U Maloney murine leukemia virus reverse transcriptase (Bethesda Research Lab, Gaithersburg, MD) in a final volume of 20 μL. After incubation at 42°C for 45 minutes, the mixture was heated at 94°C for 5 minutes and placed on ice.

PCR was used to amplify specific DNA fragments identified by the synthesized oligonucleotide primers. The reaction mixture contained 10 mmol/L Tris-HCl buffer pH 8.0, 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, 200 μmol/L dNTPs, 1 μmol/L each of the 5' and 3' oligonucleotide primers, and 200 U Maloney Murine leukemia virus reverse transcriptase (Bethesda Research Lab, Gaithersburg, MD) in a final volume of 20 μL. After incubation at 42°C for 45 minutes, the mixture was heated at 94°C for 1 minute, primer annealing at 63°C for 2 minutes, and extension at 72°C for 3 minutes. Unless otherwise indicated, 30 cycles were performed. An aliquot (10 μL) of the PCR amplified mixture was electrophoresed in a 1% agarose gel and hybridized with an appropriate DNA probe labeled with [32P] dCTP.

**Northern analysis.** Total RNA from 10^8 PMN (approximately 5 μg/lane) was electrophoresed on agarose/formaldehyde gels and transferred to nylon membranes (Hybond-N; Amersham). Probes were PCR products of the specific genes (gp91-phox, CD64), precipitated with ammonium acetate and labeled with [32P] dCTP, random hexamers, and Klenow fragment of E. coli DNA polymerase I. The membranes were prehybridized with 5X SSC, 5X Denhardt's solution, 1% SDS, 0.2 mg/mL Torula RNA in phosphate buffer pH 7.0, wrapped and exposed to x-ray film (Kodak XARS, Rochester, NY).

**Fluorometric assay of PCR products.** Hoechst 33258 binds to double-stranded DNA in a sequence-dependent manner and could be used for the quantitative detection of amplified double-stranded DNA sequences in the PCR mixture. The fluorescent assay was performed as described. Hoechst 33258 was kept as a 1 mg/mL stock solution at 4°C in the dark. A 20-μL aliquot of the PCR product was added to 0.5 mL of a working solution containing 0.1 μg/mL of Hoechst 33258 in 10 mmol/L Tris-HCl buffer pH 7.4, 1 mmol/L Na2EDTA, and 0.1 mmol/L sodium chloride prepared just before use. Fluorescence was determined using a Perkin-Elmer LS-5 Fluorescence Spectrophotometer using an excitation wavelength of 348 nm and an emission wavelength of 470 nm. The average yield of RNA in different donors varied from 12 to 18 μg/10^6 cells. The PCR mixture was overlaid with mineral oil and subjected to PCR amplification using a Perkin Elmer Thermal cycler (Norwalk, CT). The amplification procedure involved denaturation at 94°C for 1 minute, primer annealing at 63°C for 2 minutes, and extension at 72°C for 3 minutes. Unless otherwise indicated, 30 cycles were performed. An aliquot (10 μL) of the PCR amplified mixture was electrophoresed in a 1% agarose gel and hybridized with an appropriate DNA probe labeled with [32P] dCTP, random hexamers, and Klenow fragment of E. coli DNA polymerase I. The membranes were prehybridized with 5X SSC, 5X Denhardt's solution, 1% SDS, 0.2 mg/mL Torula RNA in phosphate buffer pH 7.0, wrapped and exposed to x-ray film (Kodak XARS, Rochester, NY).

**Statistical analysis.** Statistical differences are determined using Student's two-tailed t-test for independent means (not significant, NS, P > .05).

**RESULTS**

*Effect of IFN-γ on respiratory burst.* Table 2 demonstrates the effect of 18- to 20-hour preincubation of PMN.

<table>
<thead>
<tr>
<th>Species</th>
<th>5' Primers</th>
<th>3' Primers</th>
<th>Size of PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91-phox</td>
<td>5'-ATGCTGTCTCTTGCAGCAGTCTCG-3'</td>
<td>5'-CTTGGTGATGACCACCTCTTG-3'</td>
<td>705</td>
</tr>
<tr>
<td>p47-phox</td>
<td>5'-ATGTCCTGGTGAGGAGGCC-3'</td>
<td>5'-CCAGACCCCTGAAGATCTCC-3'</td>
<td>720</td>
</tr>
<tr>
<td>p67-phox</td>
<td>5'-ATGGGAGACCCTTCTCAGG-3'</td>
<td>5'-GGATCAGCGCTTGCTTG-3'</td>
<td>616</td>
</tr>
<tr>
<td>CD64 (FcRI)</td>
<td>5'-ATGGTGTTCTGACAACCTCG-3'</td>
<td>5'-CCAGTGAAAACATTTAAGGC-3'</td>
<td>445</td>
</tr>
<tr>
<td>CD32/PC (FcRII/PC)</td>
<td>5'-ATGTCCAGAAGTATGCTTCGCC-3'</td>
<td>5'-CAAACGGGAGAAATTCTCGG-3'</td>
<td>486</td>
</tr>
<tr>
<td>CD32/TC (FcRII/TC)</td>
<td>5'-ATGGAAACCTTCTGATTCTAAT-3'</td>
<td>5'-CCGAGACGGGAAAAATTCTTG-3'</td>
<td>511</td>
</tr>
<tr>
<td>CD16 (FcRIII)</td>
<td>5'-GCTGGTGGAGAAGGACAGTG-3'</td>
<td>5'-ATGTCCAGACCTCTGGATGCTG-3'</td>
<td>457</td>
</tr>
<tr>
<td>CD18 (CR3-β chain)</td>
<td>5'-ATCGGTCAGCCTACCTCGTG-3'</td>
<td>5'-TCATGGTGGAGAAGGACAGTG-3'</td>
<td>488</td>
</tr>
<tr>
<td>CD11b (CR3-a chain)</td>
<td>5'-GTGTTTATTTACATACCCAGG-3'</td>
<td>5'-CTCCTAATGTCGAGCAGATTC-3'</td>
<td>548</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GTGGGCGGCCTCAGCCAGCACC-3'</td>
<td>5'-CTCCTAATGTCGAGCAGATTC-3'</td>
<td></td>
</tr>
</tbody>
</table>
with and without IFN-γ on their response to a 1-hour exposure to either TNF, GM-CSF, formylmethionyl-leucyl-phenylalanine (FMLP), opsonized zymosan, or PMA. Two aspects of the respiratory burst, iodination and O₂⁻ production, were measured. In every instance, with the exception of O₂⁻ production after GM-CSF addition to IFN-γ-treated PMN, the stimulant significantly increased iodination and O₂⁻ production (Table 2), with the stimulation by opsonized zymosan or PMA generally being greater than the stimulation by TNF, GM-CSF, or FMLP. Iodination and O₂⁻ production by restim PMN or PMN stimulated by TNF, GM-CSF, FMLP, or opsonized zymosan was significantly greater after an 18- to 20-hour preincubation with IFN-γ than without IFN-γ; however, stimulation by PMA was unaffected by IFN-γ pretreatment (Table 2, P*). The increased iodination after preincubation with IFN-γ was prevented by the addition to the preincubation medium of a neutralizing rabbit antibody to human IFN-γ (Table 2).

Iodination by freshly isolated PMN was unaffected by exposure for 1 hour to LPS at concentrations ranging from 0.01 to 50 µg/mL under conditions used in Fig 1. Similarly, PMN preincubated for 18 to 20 hours in culture medium alone did not respond to LPS with increased iodination. However, when the PMN were preincubated for 18 to 20 hours with 100 U/mL IFN-γ, there was a small but significant increase in baseline iodination, which was further increased by LPS. The effect of LPS was maximal at a concentration of 5 µg/mL. The baseline iodination and the stimulation of iodination by LPS seen with IFN-γ-pretreated PMN was completely prevented by polymyxin B, which binds to LPS and inhibits its action.

**Effect of IFN-γ on bactericidal activity.** The effect of preincubation with and without IFN-γ on the bactericidal activity of PMN is shown in Fig 2. Fresh PMN produce approximately a 2 log decrease in S aureus viable cell count over the 90-minute incubation period under the conditions used. PMN preincubated without IFN-γ for 18 to 20 hours had significantly reduced bactericidal activity that was partially prevented by the addition of IFN-γ to the preincubation medium.

**Effect of IFN-γ on adherence.** Table 3 demonstrates the effect of preincubation for 18 to 20 hours in the presence and absence of IFN-γ on the adherence of PMN to plastic. A 1-hour incubation with PMA, FMLP, or TNF significantly increased the adherence of fresh PMN, whereas the increase observed with GM-CSF or LPS was not significant (P > 0.05) under the conditions used. Adherence of the PMN was significantly depressed after overnight storage in the absence of IFN-γ, with PMA, FMLP, TNF, or LPS as the stimulus, and this depression was partially or com-

### Table 2. Effect of Preincubation With IFN-γ on PMN Response to a Number of Stimuli

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>-IFN-γ</th>
<th>+IFN-γ</th>
<th>P*</th>
<th>+IFN-γ +aIFN-γ</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.3 ± 0.5 (19)</td>
<td>62.4 ± 12.3 (21)</td>
<td>&lt;.001</td>
<td>1.9 ± 1.1 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>TNF</td>
<td>47.3 ± 9.5 (13)</td>
<td>456.8 ± 53.9 (19)</td>
<td>&lt;.001</td>
<td>20.3 ± 2.9 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>87.0 ± 14.9 (13)</td>
<td>190.2 ± 27.3 (19)</td>
<td>&lt;.01</td>
<td>102.5 ± 18.4 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>FMLP</td>
<td>69.8 ± 10.2 (12)</td>
<td>257.0 ± 27.3 (18)</td>
<td>&lt;.01</td>
<td>83.9 ± 1.8 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>Ops. Zym</td>
<td>1002.2 ± 87.8 (12)</td>
<td>1553.4 ± 83.7 (18)</td>
<td>&lt;.001</td>
<td>1014.1 ± 169.8 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>PMA</td>
<td>1203.0 ± 110.3 (12)</td>
<td>972.1 ± 108.8 (18)</td>
<td>&lt;.01</td>
<td>1567.4 ± 254.7 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>P*</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

For measurement of iodination, the reaction mixture contained the standard salt solution, 8 x 10⁻⁵ mol/L sodium iodide (4 nmol; 0.05 µCi ¹²⁵I), 0.25 mg albumin, either 1 x 10⁵ PMN preincubated without IFN-γ (-IFN-γ), 1 x 10⁵ PMN preincubated with 100 U IFN-γ/mL (+IFN-γ), or 1 x 10⁵ PMN preincubated with IFN-γ + antihuman IFN-γ (+IFN-γ + aIFN-γ), and where indicated 100 U/mL LPS, 1 µg/mL GM-CSF, 10⁻⁴ mol/L FMLP, 1 mg/mL opsonized zymosan and 10 ng/mL PMA. For measurement of O₂⁻ production, the reaction mixture was as described above, except that ¹²⁵I-iodide and albumin were omitted, 2 x 10⁻⁴ mol/L ferricytochrome C was added, and the final volume was 1.0 mL.

*P values for the difference from PMN preincubated in the absence of IFN-γ (-IFN-γ).
†Mean ± SE of n experiments.
‡P values for the difference from the absence of stimulant.
EFFECT OF IFN-γ ON NEUTROPHIL FUNCTION

8

four to eight experiments. The reaction mixture contained the standard salt solution and either 3 to 4 x 10^6 freshly isolated PMN activity. The reaction mixture contained the standard salt solution and either 3 to 4 x 10^6 freshly isolated PMN (Δ—Δ), S. aureus + 5 x 10^6 freshly isolated PMN (●—●), S. aureus + 5 x 10^6 PMN preincubated 18 hours without IFN-γ (○—○), and S. aureus + 5 x 10^6 PMN preincubated 18 hours with 100 U/mL IFN-γ (□—□). The P values for the difference between preincubation with and without IFN-γ is shown. Incubation was for the periods indicated. The results are the mean of four to eight experiments.

Fig 2. Effect of preincubation of PMN with IFN-γ on bactericidal activity. The reaction mixture contained the standard salt solution and either 3 to 4 x 10^6 S. aureus alone (Δ—Δ), S. aureus + 5 x 10^6 freshly isolated PMN (●—●), S. aureus + 5 x 10^6 PMN preincubated 18 hours without IFN-γ (○—○), and S. aureus + 5 x 10^6 PMN preincubated 18 hours with 100 U/mL IFN-γ (□—□). The P values for the difference between preincubation with and without IFN-γ is shown. Incubation was for the periods indicated. The results are the mean of four to eight experiments.

Table 3. Effect of Preincubation of PMN With IFN-γ on Adherence

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Fresh PMN</th>
<th>PMN Scored 18 h Without IFN-γ</th>
<th>PMN Scored 18 h With IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.1 ± 2.5 (Mb)</td>
<td>3.1 ± 0.9 (7)</td>
<td>12.1 ± 4.2 (7)</td>
</tr>
<tr>
<td>PMA</td>
<td>64.5 ± 1.3 (4)</td>
<td>23.1 ± 2.0 (7)</td>
<td>36.5 ± 3.5 (7)(†)</td>
</tr>
<tr>
<td>FMLP</td>
<td>35.6 ± 6.2 (4)</td>
<td>10.1 ± 2.9 (5)</td>
<td>22.4 ± 3.7 (5)(*)</td>
</tr>
<tr>
<td>TNF</td>
<td>23.3 ± 5.0 (4)</td>
<td>7.1 ± 2.0 (7)</td>
<td>20.4 ± 5.0 (7)(†)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10.7 ± 4.1 (4)</td>
<td>2.6 ± 1.1 (6)</td>
<td>11.7 ± 5.5 (6)</td>
</tr>
<tr>
<td>LPS</td>
<td>13.6 ± 4.3 (4)</td>
<td>3.3 ± 1.4 (7)</td>
<td>19.4 ± 6.3 (7)(†)</td>
</tr>
</tbody>
</table>

To each of quadruplicate wells was added 4 x 10^6 PMN in 100 μL of HBSS. The PMN were either fresh, stored for 18 hours without IFN-γ, or stored for 18 hours with 100 U/mL IFN-γ. Where indicated, 10 ng/mL PMA, 10^-7 mol/L FMLP, 100 U/mL TNF, 1 μg/mL GM-CSF, or 5 μg/mL LPS was added and adherence was determined after a 1-hour incubation period.

*Mean ± SE of (n) experiments.
†Significantly different from fresh PMN P < .05.
‡Significantly different from PMN stored 18 hours without IFN-γ P < .05.

Effect of IFN-γ on surface receptor expression. Figure 3 demonstrates the effect of the incubation of PMN with IFN-γ for periods up to 48 hours on the surface expression of CD11b (CR3 α-chain), CD18 (CR3 β-chain), CD64 (FcRI), CD32 (FcRII), and CD16 (FcRIII). Fresh PMN contain a high density of CD11b, CD18, CD32, and CD16, but lack perceptible CD64 on their surface. With a short incubation (30 minutes) at 37°C, an increase in the surface expression of CD11b and CD18 was observed; however, when the incubation was extended, a time-dependent decrease in the fluorescence intensity was observed with FITC-labeled antibodies directed against CD11b, CD18, CD32, and CD16. In a number of instances, there was evidence of two or more populations of PMN differing in the density of their antigen expression. Incubation of PMN with IFN-γ decreased the rate of fall in surface CD11b, CD18, CD32, and CD16 expression with time. In contrast, CD64, normally undetectable on the PMN surface, was expressed after 24- and 48-hour incubations of the cells with IFN-γ.

Effect of IFN-γ on mRNA levels. mRNA for gp91-phox, p67-phox, p47-phox, CD64, CD32/PC, CD32/TC, CD16, CD11b, CD18, and actin were detected in freshly isolated neutrophils from all donors using PCR amplification of neutrophil RNA-derived cDNA recognized by synthetic oligonucleotides specific for each protein (Figs 4 and 5). In each case, culture of the neutrophils for 18 to 20 hours in the absence of IFN-γ resulted in a decrease in specific mRNA as indicated in gels stained with ethidium bromide or with Hoechst 33258 for phagocyte oxidase components, and by quantitative measurement by Hoechst 33258 fluorescence. When IFN-γ was added to the culture medium, the fall in mRNA was decreased or prevented in all instances, except for CD18, where the decline in mRNA levels during overnight culture was unaffected by IFN-γ. In contrast, addition of IFN-γ increased gp91-phox mRNA to levels greater than that of freshly isolated neutrophils. This was observed with cells of six separate donors. With some donors, mRNA for CD64 was increased by culture with IFN-γ to levels greater than that of the freshly isolated neutrophils; however, this result was not observed with other donors (Fig 5). Of the various specific mRNAs measured, the mRNA for CD64 was the lowest following culture without IFN-γ. With CD32/PC mRNA, the decrease was not large but was consistently observed with the five donors. The differences in mRNA levels between freshly isolated neutrophils and neutrophils cultivated with and without IFN-γ were confirmed by 32P oligonucleotide hybridization studies using p47-phox PCR-amplified cDNA (Fig 6). PCR amplification for 18, 22, and 26 cycles was performed. No hybridization was detected with material amplified for 18 cycles (data not shown). However, autoradiograms of PCR product amplified for 22 and 26 cycles showed a pattern comparable with that of ethidium bromide- or Hoechst 33258-stained gels. Restriction endonuclease digestion of the PCR products yielded the expected molecular weight fragments (Fig 7).

Northern analysis confirmed the PCR findings for the two mRNAs studied (Fig 8). With three of three donors, mRNA for p47-phox fell when PMNs were stored overnight without IFN-γ, and this decrease was in part or totally
RESULTS

MRNA for CD64 was detected in freshly isolated PMN from four of five donors (Fig 8), decreased to undetectable levels in PMN incubated for 18 hours without IFN-γ, and increased to levels higher than those of freshly isolated PMN after 18 hours of incubation with IFN-γ. In the fifth donor, no CD64 mRNA was detected in freshly isolated PMN or PMN incubated without IFN-γ, but increased to high levels after incubation with IFN-γ.

A PCR method was used to detect specific mRNAs in neutrophils. Up to 20 different mRNAs could be detected from 1 to 2 μg of total neutrophil RNA by this method and their relative abundance compared. Quantitation of PCR product was performed by staining double-stranded DNA by the bisbenzimidazole fluorescent dye Hoechst 33258. Our findings support recent suggestions that the PMN is a transcriptionally active cell. For all the messages studied (actin, gp91-phox, p47-phox, p67-phox, CD11b, CD18, CD64, CD32, and CD16), the steady-state level of mRNA decreased when neutrophils were stored overnight, suggesting a generalized phenomenon, which may be related to increased degradation and/or decreased transcription. With the exception of CD18, the decrease in mRNA level on storage was partially or totally prevented by IFN-γ. Both CD64 and gp91-phox mRNA increased after overnight storage of neutrophils with IFN-γ, to levels greater than those of freshly isolated neutrophils. In prior studies using Northern analysis, the steady-state level of CD64 mRNA was below the level of detection in freshly isolated PMN, but was markedly increased in cells exposed to IFN-γ. We could detect CD64 mRNA in our freshly isolated cells by both the PCR method and, in four of five individuals, by Northern analysis. It is of interest that the mRNA for CD64 was detected in freshly isolated neutrophils despite the absence of the protein on the cell surface. The CD64 mRNA decreased to undetectable levels after overnight storage in the absence of IFN-γ and generally increased to levels greater than those of freshly isolated PMN on storage with IFN-γ.

In a recent study, IFN-γ was reported to increase the

DISCUSSION

This study has confirmed and extended previous reports on the increased functional activity of PMN preincubated with IFN-γ. When compared with PMN similarly preincubated in the absence of IFN-γ, preincubation of PMN overnight (18 to 20 hours) in RPMI-FCS containing IFN-γ (100 U/mL) significantly increased their subsequent metabolic response (as measured by iodination or O2- production) to a 1-hour incubation in the absence of a stimulus or in the presence of TNF, GM-CSF, FMLP, opsonized zymosan, or LPS. In contrast, the respiratory burst induced by PMA was unaffected by pretreatment of the PMN with IFN-γ under our experimental conditions. The decrease in bactericidal activity on overnight storage of PMN was also partially prevented by IFN-γ, as was the storage-induced decline in adherence of stimulated PMN to a plastic surface. In contrast to respiratory burst activity, adherence of stored PMN stimulated with PMA was increased by IFN-γ pretreatment. The priming of PMN by IFN-γ as measured by iodination was abolished by anti-IFN-γ antibody.

The adherence of PMN to a plastic surface or to endothelial cells is dependent in large part on the PMN surface expression of the CD11b/CD18 integrin. Flow cytometric analysis of CD11b and CD18 on PMN showed a progressive loss of surface expression on culture of the PMN for 24 to 48 hours, an effect that was partially prevented by the presence of IFN-γ in the culture medium. After long-term culture, particularly with IFN-γ, two populations of PMN were detected that varied in their surface expression of the CD11b/CD18 antigens. It is probable that the decreased adherence of stored PMN which is partially prevented by IFN-γ is a consequence of these changes in the surface expression of the CD11b/CD18 complex. The loss of CD32 (FcRII) and CD16 (FcRIII) from the surface of stored PMN was also prevented, in part, by IFN-γ. In contrast, as previously reported, CD64 (FcRI) was not detected on the surface of freshly isolated PMN or PMN stored in the absence of IFN-γ, but was detected on PMN preincubated with IFN-γ.
EFFECT OF IFN-γ ON NEUTROPHIL FUNCTION

Fig 4. Effect of IFN-γ on mRNA levels of phagocyte oxidase components. The PCR product in the gel is for cDNA prepared from 2.5 x 10⁶ PMN from a single donor. The PMN were freshly isolated (designated ○), cultured 19 hours in the absence of IFN-γ (designated -), or cultured 19 hours in the presence of IFN-γ (designated +). PCR products were actin, gp91-phox, p67-phox, and p47-phox. Gel A was stained with ethidium bromide and gel B was stained with Hoechst 33258. The bar graph is a quantitative analysis of the PCR products using Hoechst 33258 fluorescence. The values were normalized to those obtained with freshly isolated PMN (100%). (■), Freshly isolated PMN; (□), PMN cultured 19 hours without IFN-γ; (△), PMN cultured 19 hours with IFN-γ. Two separate donors 1 and 2 were used.

steady-state level of mRNA for gp91-phox, to have no effect on the mRNA level of p22-phox (the small subunit of cytochrome b558), and to decrease the mRNA level of p47-phox.52 Incubation of PMN without IFN-γ (control) was for 2 hours and incubation with IFN-γ was for periods ranging from 0.5 to 20 hours. In our study, incubation with and without IFN-γ was for 18 to 20 hours and, under these conditions, we confirm that mRNA for gp91-phox is increased when compared to freshly isolated neutrophils and particularly to neutrophils incubated without IFN-γ for a comparable period. Although the p47-phox mRNA level in neutrophils incubated for 20 hours with IFN-γ was generally lower than that of freshly isolated neutrophils in our studies, it was consistently higher than that of cells incubated for a comparable period in the absence of IFN-γ. This was confirmed in three of three individuals by Northern analysis.

Because contaminating eosinophils and monocytes could theoretically contribute specific mRNA to our extract, care was taken to exclude these cells from our neutrophil preparation. The initial Histopaque procedure separates granulocytes from mononuclear cells. A second separation procedure, centrifugation over a Metrizamide gradient, was added to separate neutrophils from eosinophils in the granulocyte fraction; this step would also serve as a second procedure for the removal of monocytes from the neutrophil preparation. The final preparation contained less than 1% eosinophils (generally <0.5%) and no detectable monocytes (<0.1%). To further exclude the participation of contaminating monocytes, we determined the mRNA levels of receptor proteins. The PCR products were actin, CD32, CD32, CD16, and CD11b. Lane S contains 1 kb standards (Bethesda Research Lab). All PCR products ran at or below the 510-bp standard. The gel was stained with ethidium bromide. Quantitative analysis of PCR products by Hoechst 33258 dye fluorescence is shown in the bar graphs. (■), Freshly isolated PMN; (□), PMN cultured 19 hours without IFN-γ; (△), PMN cultured 19 hours with IFN-γ. Two separate donors 1 and 2 were used.
Fig 6. Quantitation of p47-phox PCR product by 32P-oligonucleotide hybridization. A probe complementary to an internal sequence of the antisense strand of p47-phox PCR amplified cDNA was labeled with 32P. The PCR products prepared from freshly isolated PMN are designated 0, from PMN cultured for 19 hours without IFN-γ are designated −, and from PMN cultured for 19 hours with IFN-γ are designated +. The PCR product was obtained after 22 or 26 cycles. The gels were stained with ethidium bromide (A) and then hybridized with the 32P-probe and an autoradiogram prepared (B). The band indicated by the arrow was excised from the agarose gel and counted in a liquid scintillation counter. The counts per minute (mean of three experiments) are shown.

Fig 8. Northern analysis of PMN mRNA for p47-phox and CD64 (FcRI). The PMN were freshly isolated (designated ○), cultured 18 hours in the absence of IFN-γ (designated −), or cultured 18 hours in the presence of IFN-γ (designated +). Staining of duplicate lanes with ethidium bromide (not shown) indicated that equivalent amounts of rRNA were present in each lane.

level for gp91-phox and p47-phox in monocytes either freshly isolated or cultured for 18 to 21 hours with or without IFN-γ (data not shown). An equivalent amount of mRNA would have required contamination of our PMN preparations by greater than 1% monocytes.

In general, IFN-γ limits the decrease in neutrophil respiratory burst and bactericidal activity, surface complement and Fc receptors, and mRNA levels on overnight storage. The maintenance of mRNA pools may be central to the more distal effects on surface protein levels and functional activity. This protective role for IFN-γ on neutrophils raises the possibility of the use of IFN-γ in the maintenance of the functional activity of stored blood or neutrophil preparations.

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Effects of gamma-interferon on human neutrophils: protection from deterioration on storage

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