Effects of Interleukin-2 on Gene Expression in Human Neutrophils

By Denis Girard, Jean Gosselin, Dominique Heitz, Robert Paquin, and André D. Beaulieu

Recently, the interleukin-2 receptor (IL-2R) was shown to be present on human neutrophils, and IL-2–neutrophil interactions are believed to be important in both tumor rejection and increased susceptibility to bacterial infections. Furthermore, neutrophils have been shown to synthesize host defense proteins, such as cytokines. In this study, we analyzed the effects of IL-2 on the induction of de novo RNA and protein synthesis in this cell type. When cells were stimulated with IL-2 alone, the level of incorporation of either [5-3H]-uridine or [35S]-methionine and [35S]-cysteine was similar to unstimulated cells. However, when cells were stimulated with the combination of a fixed concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF), a dose-dependent effect of IL-2 was observed on the induction of both RNA and protein synthesis. In the presence of tumor necrosis factor-alpha and formyl-methionyl-leucyl-phenylalanine, however, IL-2 exerted no similar effect. Furthermore, the study of a large number of normal subjects (n = 55) showed reproducible categories of responders (low, intermediate, and high). The binding of IL-2 to the IL-2R complex on human neutrophils increased on GM-CSF–stimulated neutrophils compared with unstimulated cells. However, no increase in the level of expression of either the α or β chains of this receptor complex was observed. This finding suggests that GM-CSF functionally activates the IL-2R, but does not regulate its level of expression. Finally, we found that human neutrophils constitutively express IL-2Rγ chain mRNA and thus have the potential to express the functional IL-2R complex. Our findings on IL-2–neutrophil interactions should lead to new avenues of research in understanding the responses of patients undergoing GM-CSF or IL-2 therapy.

© 1995 by The American Society of Hematology.

MATERIALS AND METHODS

Neutrophil isolation. Neutrophils were isolated from venous blood obtained from normal volunteers by centrifugation over Ficoll-Hypaque (Pharmacia Biotech Inc, Quebec, Canada) as previously described. Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. All cell suspensions contained fewer than 1% monocytes as determined by monocyte staining, and cell viability was always greater than 99%.

Cytokines. Recombinant human IL-2 (specific activity of 22 × 10^6 U/mg) was provided by Cetus Corporation (Emeryville, CA). Recombinant human GM-CSF (specific activity of 9 × 10^6 U/mg) was a gift from the Genetics Institute (Boston, MA).

RNA synthesis assay. This assay was performed by measuring the incorporation of [5-3H]-uridine (Amersham Corp, Oakville, Ontario, Canada) into total RNA essentially as previously described. One hundred microliters of a 5-x-10^6-cell/mL suspension in RPMI with 1% autologous serum was incubated in 96-well microtiter plates in the presence of 1 μCi of [5-3H]-uridine along with cytokines for 4 hours at 37°C, 5% CO2 in triplicate. This time point had previously been shown to be optimal. Cells were then collected onto borosilicate glass fiber paper by a multiple cell culture harvester (Skatron Instruments Inc, Sterling, VA), and sections of the filter corresponding to each microwell were then punched out and placed in scintillation vials in the presence of 4 mL of Aquasol-2 (Dupon New England Nuclear, Boston, MA). Results are expressed as mean cpm (±SE) obtained from studying several normal subjects. Stimula-
RNA synthesis was studied in neutrophils by measuring stimulation indices, which represent the ratios of counts per minute obtained with stimulated over unstimulated neutrophils.

**Metabolic labeling of neutrophils, protein precipitation and two-dimensional gel electrophoresis.** Metabolic labeling of neutrophils (1 x 10^7 cells) was performed with [35S]-methionine and [35S]-cysteine (Amersham), at both 125 μCi/10^7 cells as previously described. The cells were incubated in RPMI 1640 medium containing 1% autologous serum in the presence of protease inhibitors (aprotinin, 60 trypsin inhibiting units/ml; phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin, 0.5 μg/ml, and EDTA 200 μmol/L). Protein precipitation was performed in Eppendorf tubes with a final concentration of 70% ethanol for 1 hour at -20°C. Intracellular and extracellular proteins were treated separately. After centrifugation of the precipitates, the pellets were solubilized with the lysis buffer (9.5 mol/L urea, 2% NP-40 and 5% β-mercaptoethanol), and 10 mL of each corresponding fraction was placed in scintillation counting vials with 4 mL of Aquasol-2. This was performed to determine the amount of radiolabeled proteins loaded for the migration. High-resolution two-dimensional gel electrophoresis was performed as previously described using the Millipore Investigator 2D Electrophoresis System (Millipore Corp, Bedford, MA). One-dimensional isoelectric focusing was performed using 2% ampholine (1-4, vol/vol, pH 4-8, and pH 3-10). Second-dimensional gels were 12% acrylamide. Gels were dried and exposed for 3 to 5 days at -70°C. Two-dimensional gel analysis of proteins was performed with the BioImage 110-S analyzer (Millipore) using the 2D Gel Match Program (Ann Arbor, MI).

**Flow cytometry.** We determined IL-2R expression by using the relative binding of Fluororine IL-2 phycocerythrin (PE) and Fluororine streptavidin-PE (S-PE) according to the manufacturer’s instructions (R&D System, Minneapolis, MN), performed with GM-CSF-treated cells (3 x 10^6 mol/L) and unstimulated cells incubated for 30 minutes at 37°C, 5% CO2. In other experiments, 1 x 10^6 neutrophils were dualy stained directly with 20 mL of PE-labeled monoclonal antibody to the human IL-2Rα chain (p73) (Becton Dickinson, Mountain View, CA) and 5 mL of fluorescein isothiocyanate-labeled antibody to the human IL-2Rβ chain of the IL-2R complex (p75) (Endogen, Boston, MA). Staining was performed according to the manufacturer’s recommended procedures. Dual-color isotypic stainings were used as negative controls (Bio/Can Scientific, Mississauga, Ontario). Final flow cytometric analysis was performed using an EPIC 753 (Coulter, Miami Lakes, FL).

**RNA isolation and northern blot analysis.** The total RNA was isolated by the RNeasy method (ID Labs, Lodon, Ontario, Canada), and 15 μg of total RNA was loaded on a 1% agarose denaturing gel and size-fractionated by electrophoresis. Gels were then visualized under ultraviolet (UV) illumination to verify that equal amounts of RNA had been loaded and also to determine the position of the 18S and 28S ribosomal RNA bands. The transfer was performed in 3 hours with the VacuGene TM XL vacuum blotting system according to the manufacturer’s specifications (Pharmacia), and was complete for each lane. The IL-2Rγ probe was obtained by polymerase chain reaction (PCR) amplification using the two following primers based on the published sequence of the mRNA: 5’ GAAGAGCAAGCOCCTATGGT 3’ and 5’ CGGCTCCGCAAACATCGGAG 3’. This step yielded a 1.4 kb fragment, which was purified on a low-melting-point agarose gel. After phosphorylation, the membranes were hybridized with random-primer 32P-labeled probes according to the Prime-a-Gene Labeling System (Promega, Madison, WI) in 50% formamide overnight at 42°C. The membranes were then washed and exposed to Kodak X-Omat films (Eastman-Kodak, Rochester, NY) with an intensifying screen at -70°C.

**RESULTS**

**Induction of de novo RNA synthesis in neutrophils.**

Total RNA synthesis was studied in neutrophils by measuring [3H]-uridine uptake. We incubated IL-2 with neutrophils and observed that the level of incorporation was similar whether the neutrophils were stimulated with IL-2 (from 10 to 10^3 U/ml) or not (Fig 1A). However, we also found that the level of incorporation increased in a dose-dependent manner when the cells were stimulated with a combination of a predetermined optimal but fixed GM-CSF concentration (3 x 10^-9 mol/L) and increasing concentrations of IL-2 (Fig 1A). Based on these data, we further compared the induction of total RNA synthesis using cells treated with GM-CSF, IL-2 (10^3 U/ml), or a combination of GM-CSF and IL-2 in a large population of normal donors (Fig 1B). We first observed that some subjects were low-responders to GM-CSF, with a stimulation index less than 3 (n = 14), whereas others were either intermediate- (n = 21) or high- (n = 20) responders with indices between 3 and 5 or above 5, respectively. Again, IL-2 alone had minimal effects on the induction of RNA synthesis in all three groups. However, IL-2 significantly enhanced the responses to GM-CSF in the intermediate- and high-responder groups, whereas the low-responders to GM-CSF were not influenced by IL-2. Results for the responder groups were reproducible when the donors were tested several times over a period of days (Table 1). The induction of total RNA synthesis was inhibited when the cells were incubated in the presence of 5 mg/mL of actinomycin D, an inhibitor of transcription (results not shown). Finally, we tested IL-2 in combination with either TNF-α or fMLP, both neutrophil agonists that markedly affect gene expression in this cell type. In contrast with the data obtained with GM-CSF, no major effect on the induction of RNA synthesis resulted from combining IL-2 with either TNF-α or fMLP (Table 2).

**Induction of de novo protein synthesis in neutrophils.**

Neutrophils were next stimulated with the combination of GM-CSF + IL-2 for 20 hours in the presence of [35S]-methionine and [35S]-cysteine. The de novo synthesized proteins were analyzed by two-dimensional gel electrophoresis and fluorography (Fig 2A and B). However, before loading on the gels, the labeled proteins were precipitated from cell supernatants and lysates with 70% ethanol, and total counts were compared with those obtained from unstimulated cells and GM-CSF- or IL-2-stimulated cells. As in the RNA synthesis studies, indices of stimulation were calculated by dividing the counts obtained with stimulated cells by counts for unstimulated cells. The indices we obtained closely paralleled those of the RNA synthesis assay (data not shown), indicating that the RNA transcription was followed by translation into proteins. The fluorograms show are representative of results obtained when studying four different subjects and were performed with cell lysates obtained from neutrophils that had been stimulated either by GM-CSF alone (Fig 2A) or by the combination of GM-CSF + IL-2 (Fig 2B). It should be noted that the results shown in Fig 2A and B were obtained from an individual categorized as an intermediate-responder to IL-2, suggesting that a relatively small amount of IL-2 is required to induce de novo protein synthesis in neutrophils. The total counts obtained from each preparation are shown at the top of the fluorograms and represent counts of lysates obtained from an equal number of stimulated neutrophils. Stimulation by GM-CSF in combination with IL-2...
Fig 1. Induction of de novo RNA synthesis in human neutrophils by IL-2 and GM-CSF. Cells were isolated from normal volunteers as described in Materials and Methods. De novo RNA synthesis was studied by measuring [5-3H]-uridine incorporation in total RNA after 4 hours of stimulation. (A) A representative dose-response experiment with cells from one donor (n = 10). Cells were either stimulated with increasing concentrations of IL-2 (□) or with the combination of a fixed concentration of GM-CSF (3 × 10⁻⁵ mol/L) and increasing concentrations of IL-2 (●). (B) Measurement of de novo RNA synthesis after stimulation for 4 hours with either GM-CSF, IL-2, or the combination of GM-CSF and IL-2 in a population of 55 different donors. Concentrations used were 3 × 10⁻⁵ mol/L for GM-CSF and 10⁶ U/mL for IL-2. Low responders: n = 14; intermediate responders: n = 21; high responders: n = 20. The asterisk indicates that responses to stimulation with the combination of GM-CSF and IL-2 were significantly greater (P < .05) than when cells were stimulated with GM-CSF alone. Statistical analysis was performed using the Student’s t-test.

gave rise to proteins that could not be visualized on the fluorogram obtained from stimulating cells with GM-CSF alone. These proteins, yet unknown, are identified by the boxes labeled a and b. However, for most proteins visualized on these gels, synthesis was enhanced by the combination of GM-CSF + IL-2. This enhancement is shown graphically by densitometry analysis of 10 randomly selected, but reproducible protein spots obtained from scanning fluorograms performed with four different blood donors (Fig 2C). All values represent the mean and standard errors of the measurements performed on all four fluorograms. Of the 10 spots selected, eight showed greater intensity in the GM-CSF + IL-2 fluorogram, two in the GM-CSF fluorogram (spots 6 and 10). Although this finding indicates a generalized increase in protein synthesis when cells are stimulated with GM-CSF + IL-2 in comparison with GM-CSF alone, the synthesis of the two proteins identified by spots 6 and 10 was inhibited by combining the two agonists. Furthermore, the combination of GM-CSF and IL-2 resulted in the stimulation of synthesis in a proportion greater for some proteins than for others. For example, the protein represented by spot 5 increased by 2.5-fold compared with a less than 0.5-fold increase for the protein represented by spot 8. Studies performed with cell supernatants yielded information similar to the above, and the results are, therefore, not shown.

IL-2R expression on neutrophils. We next compared, by flow cytometry, expression of the IL-2R complex on unstimulated and GM-CSF-stimulated neutrophils using monoclonal antibodies to the human α and β chains of the IL-2R complex (Fig 3). As previously reported, we observed that the α chain was undetectable (<1% of positive cells) and that ~70% of cells were positive for the β chain. The mean log fluorescence intensity measured with unstimulated and GM-CSF-stimulated cells was quite stable for both chains. These findings show that GM-CSF treatment of neutrophils does not affect the proportion of neutrophils expressing IL-2R or the level of expression.

We next compared IL-2 binding on unstimulated and GM-CSF-stimulated neutrophils using flow cytometry (Fig 3). Although the β chain of the IL-2R complex is expressed on more than 70% of neutrophils, fewer than 20% of unstimulated neutrophils bind IL-2. However, after GM-CSF stimu-
The population of neutrophils capable of binding IL-2 was increased to greater than 40%, representing a mean increase of 54% in the population of GM-CSF-stimulated neutrophils capable of binding IL-2. Binding of PE-labeled IL-2 to unstimulated and GM-CSF-stimulated neutrophils was inhibited when cells were preincubated with unlabeled IL-2, showing the specificity of the flow cytometry measurements for binding to the IL-2R (Fig 4). It should be noted that the expression of the IL-2R on only 40% of the cells does not necessarily mean that we have identified a subpopulation of IL-2 positive neutrophils. This is based on the fact that in a population of homogenous activated B lymphocytes, the PE-labeled IL-2 reagent that we have used reacted with approximately the same percentage of cells. We have used reacted with approximately the same percentage of cells. This suggests that binding of the reagent is the limiting factor and therefore, the conclusion that there exists a subpopulation of IL-2-positive neutrophils should not be made at this time.

Constitutive expression of the IL-2Rγ chain in neutrophils. We next investigated the presence of the IL-2Rγ chain of the IL-2R complex in neutrophils. This chain is essential, along with the b chain, to IL-2R activity. As no antibody to this chain was available, we used Northern blots to study if the mRNA corresponding to this component of the IL-2R complex is present in neutrophils, and we found that neutrophils expressed and accumulated mRNA for this protein (Fig 5) suggesting that this protein is present or has the potential to be present on the surface of these cells. However, accumulation of mRNA was not affected by stimulation of neutrophils with GM-CSF. This is in agreement with the recent work of Liu et al. Although we show only the 4-hour time point, a complete time course study at 30 minutes, 1 hour, 4 hours, and 16 hours was also performed, and no evidence of modulation of mRNA levels was obtained (results not shown).

**DISCUSSION**

This study, in which we analyzed the effects of IL-2 on modulating gene expression in neutrophils, was considered important for a number of reasons. First, patients undergoing IL-2 therapy for cancer show an increased susceptibility to bacterial infections, and the functional responses of neutrophils are negatively affected by this form of treatment.

Second, the antitumoral effects of IL-2 are believed to be partly mediated by neutrophils. Third, it is now recognized that neutrophils synthesize de novo a number of host-defense proteins when participating in both the afferent and efferent limbs of the immune response. In all these instances, there is little understanding of the mechanisms involved, and the precise role of IL-2 remains undetermined. Therefore, we decided to study the effects of this cytokine on de novo RNA and protein synthesis in neutrophils with the hope of shedding additional light on the mechanisms by which this important immunomodulating molecule can influence neutrophil functions.

Although IL-2 has already been shown to induce de novo synthesis of TNF-α and IL-8 by neutrophils, we had previously observed that this interleukin exerts only weak activity in initiating the nuclear signaling events that are necessary for the general activation of the RNA and protein synthesis machinery in this cell type. We demonstrated that GM-CSF, along with TNF-α and FMLP, are the only neutrophil agonists of a large array tested that possess the capacity to modulate significantly gene expression in this phagocyte. However, we now report that such an effect is observed with IL-2, but only when neutrophils are also stimulated by GM-CSF. When the incorporation of [5-3H]-uridine into total RNA was used to measure de novo RNA synthesis, IL-2 exerted a dose-dependent effect on the induction of de novo RNA synthesis in GM-CSF-treated neutrophils (Fig 1A), but it remained inactive on unstimulated or TNF-α- and FMLP-stimulated cells (Table 2). Furthermore, previous reports on the induction of TNF-α and IL-8 production by IL-2 pointed out that the amount of production for these cytokines varied considerably among subjects. We obtained similar results studying the induction of de novo RNA synthesis and have examined this phenomenon in great detail. First, we studied 55 different normal subjects and observed three distinct groups of responders to GM-CSF (Fig 1B). We refer to them as low-, intermediate-, or high-responders based on the stimulation indices obtained with their neutrophils. Second, IL-2 increased this response to levels that were significantly greater than those observed with cells treated with GM-CSF alone. Although levels were increased by IL-2, subjects remained in the same category of response. These results strongly suggested that neutrophils isolated from different subjects vary in their responsiveness to these cytokines. However, before coming firmly to this conclusion, we conducted a study to test the reproducibility of our results. We randomly chose 12 subjects who were available for repeated testing on different days. The results showed that subjects remained essentially in the same categories of response after...

---

**Table 1. Reproducibility of Results for Each Group of Responders After Stimulation of Human Neutrophils With GM-CSF**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Index ± SE</th>
<th>No. of Repeats</th>
<th>Type of Responder</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.8 ± 0.6</td>
<td>2</td>
<td>High</td>
</tr>
<tr>
<td>2</td>
<td>6.3 ± 0.6</td>
<td>2</td>
<td>High</td>
</tr>
<tr>
<td>3</td>
<td>5.7 ± 0.4</td>
<td>6</td>
<td>High</td>
</tr>
<tr>
<td>4</td>
<td>5.5 ± 0.2</td>
<td>4</td>
<td>High</td>
</tr>
<tr>
<td>5</td>
<td>5.5 ± 0.4</td>
<td>3</td>
<td>High</td>
</tr>
<tr>
<td>6</td>
<td>5.1 ± 0.5</td>
<td>2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>7</td>
<td>3.2 ± 0.5</td>
<td>5</td>
<td>Intermediate</td>
</tr>
<tr>
<td>8</td>
<td>2.9 ± 0.1</td>
<td>2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>9</td>
<td>2.8 ± 0.3</td>
<td>2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>10</td>
<td>1.9 ± 0.6</td>
<td>3</td>
<td>Low</td>
</tr>
<tr>
<td>11</td>
<td>1.8 ± 0.2</td>
<td>3</td>
<td>Low</td>
</tr>
<tr>
<td>12</td>
<td>1.6 ± 0.3</td>
<td>4</td>
<td>Low</td>
</tr>
</tbody>
</table>

* Total RNA synthesis assay as described in Materials and Methods.

**Table 2. Induction of De Novo RNA Synthesis in Human Neutrophils After Stimulation by IL-2 in Combination With Either FMLP or TNF-α**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>Without IL-2</th>
<th>With IL-2</th>
<th>No. of Donors</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMLP</td>
<td>10 nmol/L</td>
<td>8.3 ± 1.2</td>
<td>9.6 ± 0.6</td>
<td>6</td>
<td>.355</td>
</tr>
<tr>
<td>TNF-α</td>
<td>100 ng/mL</td>
<td>6.4 ± 0.6</td>
<td>6.8 ± 1.0</td>
<td>7</td>
<td>.738</td>
</tr>
</tbody>
</table>
Fig 2. Induction of de novo protein synthesis in human neutrophils by IL-2 and GM-CSF. Cells were metabolically labeled with $[^{35}S]$-methionine and $[^{35}S]$-cysteine over a period of 20 hours. High-resolution two-dimensional gel electrophoresis and fluorography were performed as described in Materials and Methods. (A) Cells stimulated with GM-CSF alone. (B) Cells stimulated with the combination of GM-CSF (3 x 10^{-7} mol/L) + IL-2 (10^5 U/mL). Boxes a and b contain the proteins that were visualized only after stimulation with the combination of GM-CSF and IL-2, and numbers 1 to 10 serve to identify 10 randomly selected, but reproducible, protein spots. (C) Densitometry analysis of the protein spots identified by the numbers 1 to 10 in (A) and (B). Values represent mean intensity (±SE) obtained after the analysis of fluorograms performed with four different donors.

analysis on several occasions (Table 1). Therefore, it will be important not only to discover the molecular events responsible for the observed differences in neutrophil responsiveness between subjects, but also to determine the clinical significance of these findings in monitoring patients who receive GM-CSF or IL-2 therapy.

Although the synthesis of RNA induced by IL-2 in GM-CSF–stimulated neutrophils was followed by a general enhancement of protein translation, it can not be determined with any degree of precision to what extent all the RNA synthesized resulted in mRNA and protein synthesis. However, protein synthesis was clearly observed after the analysis of two-dimensional gels and the scanning of the fluorograms obtained with these gels (Fig 2). In addition to this general enhancement, selective proteins were also either induced or inhibited by the combination of GM-CSF and IL-2. Therefore, gene expression is not only enhanced, but also modulated. Furthermore, as it was previously reported that production of TNF-α and IL-8 was induced by IL-2,9,10 we measured their production by enzyme-linked immunosorbent assay (ELISA) in both the intracellular and secreted forms under our experimental conditions, and found no further induction or inhibition by the GM-CSF and IL-2 combination compared with IL-2–stimulated cells (unpublished data). This finding also argues in favor of selective modulation of gene expression when these cytokines are combined. Because GM-CSF and IL-2 are likely to be present simultaneously at sites of inflammation in vivo, our findings are biologically relevant and need to be explored fully as to their significance. In the future, it will be important to identify the nature of the proteins affected by IL-2 and to characterize their effects on neutrophil functions.

We next studied IL-2R expression and the binding of IL-2 to its receptor on GM-CSF–stimulated cells in an attempt
to characterize some of the events that might explain our findings. We first confirmed that the α chain of the IL-2R complex is undetectable on human neutrophils while the β chain is present (Fig 3). However, the levels of expression of these molecules were not influenced by GM-CSF stimulation of the cells, suggesting that an increase in the number of IL-2R expressed on these cells is not the mechanism by which neutrophils are rendered more responsive to IL-2 by GM-CSF. However, the binding of IL-2 to this receptor was clearly increased by GM-CSF stimulation, suggesting that functional activation of the receptor, rather than upregulation, is at play (Fig 4). Finally, it has been reported that, for the IL-2R complex to be functional, the recently identified γ chain of this receptor must be present along with the β chain.26-31 By Northern blots, we were able to show that human neutrophils express constitutively the γ chain mRNA and thus have the potential of expressing a functional IL-2R complex as recently described.25

We conclude that by stimulating neutrophils with GM-CSF, but not with TNF-α or FMLP, the βγ receptor complex expressed on these cells is functionally more active in binding IL-2. The increased binding of this cytokine to its receptor results in a general enhancement of gene expression in neutrophils along with the modulation of synthesis of selective proteins. Our findings may form the basis for studies...
that could lead to a better understanding of IL-2–neutrophil interactions.

REFERENCES


32. Voss SD, Hong R, Sondel PM: Severe combined immunodeficiency, interleukin-2 (IL-2), and the IL-2 receptor: Experiments of nature continue to point the way. Blood 83:626, 1994
Effects of interleukin-2 on gene expression in human neutrophils

D Girard, J Gosselin, D Heitz, R Paquin and AD Beaulieu