Expression of Interleukin-2 Receptor γ Chain on Human Neutrophils

By Jin Hong Liu, Sheng Wei, DeWayne Ussery, P.K. Epling-Burnette, Warren J. Leonard, and Julie Y. Djeu

The interleukin-2 (IL-2) receptor γ is an indispensable functional component of IL-2, IL-4, and IL-7 receptors, and thus, is denoted the common γ chain, γc. The present study was undertaken to determine whether human polymorphonuclear neutrophils (PMNs) expressed γc chain. Reverse transcription-polymerase chain reaction and Northern blot analysis showed that fresh human PMN constitutively expressed a remarkable level of γc mRNA, which is of the size and intensity of that from the peripheral blood mononuclear cells (PBMCs). Granulocyte macrophage-colony stimulating factor, IL-2, and IL-8, which are known to activate PMN functions, failed to regulate the γc gene expression. Western blot analysis with a rabbit anti-γc polyclonal antibody identified 64-, 58-, and 50kD γc bands in lysates from PMN, but only 64- and 58kD bands from PBMCs. After the PMNs and PBMCs were treated with tunicamycin to prevent N-linked glycosylation, Western blot analysis detected a single 39-kD band, which is equal to the calculated molecular weight from the cloned cDNA. Thus, our results indicate that PMNs constitutively express high levels of γc, and the three forms detected are caused by different glycosylation of a protein translated from a single mRNA species.

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INTERLEUKIN-2 (IL-2) plays a central role in the clonal expansion of activated T-cells with specific cell receptors. The IL-2/IL-2 receptor (IL-2R) system has been widely studied in lymphocytes, but it is becoming apparent that IL-2 can mediate multiple biologic functions in non-lymphoid cells, including activation of monocytes and granulocytes. These observations suggest that IL-2 is a principal regulator among immune cells and IL-2 delivers various molecules including the FcRγ. The IL-2R is unique among growth factor receptors in that it is made up of at least three distinct membrane components: IL-2Ra, IL-2Rβ, and γc, which is a newly identified peptide chain unrelated to any known membrane molecules including the FcRγ. Different combinations of these three distinct chains dictate the affinity of the IL-2R. Whereas low-affinity receptors contain IL-2Ra chain, intermediate-affinity IL-2R contain IL-2Rβ and γc chains. High-affinity receptors contain all three chains. γc is encoded by the gene that is defective in X-linked severe combined immunodeficiency and has also been shown to be a functional component of IL-4 and IL-7 receptors.

Polymorphonuclear neutrophils (PMNs) are one of the principal cellular components of host defense and are major effector cells against pathogenic microbes and in inflammatory response. The generation of a variety of mRNA and proteins relevant to their effector functions and the release of granule enzymes by these phagocytes constitute an important part of their armory designed to defeat invading microbes. Activated PMNs are also involved in the pathogenesis of tissue damage in certain inflammatory diseases such as rheumatoid arthritis. PMNs function and recruitment to the site of inflammation have been shown to be upregulated by various cytokines, including IL-1, IL-8, tumor necrosis factor (TNF), interferon γ (INFγ), and granulocyte macrophage-colony stimulating factor (GM-CSF). PMNs have also been shown to be the major cells responsible for tumor regression in mice after cytokine gene therapy with G-CSF or IL-2 gene-transfected cells. Histology of the regressing tumors indicated that rejection of the IL-2-transfected tumor cells was associated with PMN infiltration, the intensity of which is directly proportional to the amount of IL-2 released. In one clinical trial, local injection of low-dose IL-2 into the tumor mass and near the draining lymph nodes of patients with advanced primary head and neck squamous carcinomas resulted in the infiltration of granulocytes into the tumor tissue, which may have partially contributed to tumor rejection. We have shown that PMNs can respond to IL-2 with increased anti fungal activity, prolonged survival in vitro, and increased IL-8 and TNFα-induced gene expression.

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The discovery of the intermediate-affinity receptor on PMNs and knowledge that heterodimerization of IL-2Rβ and γc is required for signaling suggested to us that γc may likely be expressed on PMNs. Therefore, we wished to explore the possibility that γc is also present in PMNs, which may contribute to the intermediate IL-2 binding affinity. Our results show that PMNs constitutively express high levels of γc mRNA and protein, and that the cytokines known to activate PMN function did not alter γc gene expression.

MATERIALS AND METHODS

Preparation of PMNs and PBMCs. Leukocyte buffy coats obtained from healthy normal volunteers, at South West Florida Blood Bank (Tampa, FL) were diluted 1:2 in PBS and centrifuged over Ficoll/Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) at 400g for 30 minutes at room temperature. The human peripheral blood mononuclear cells (PBMCs) layer was collected and washed twice and used in experiments. The PMN layer lying on the surface of the erythrocyte cell pellet was collected and lysed free of contaminating erythrocytes by hypotonic shock with sterile distilled water for 30 seconds at room temperature. The cells were washed twice in PBS before adjusting to the desired cell concentration. Careful washing of the PMN preparations, such as centrifugation at 200g, aspiration

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of supernatants followed by gentle resuspension with a pipet, and avoidance of sudden changes in temperature, allowed us to avoid clumping and to maintain the viability of PMNs for up to 24 hours. PMNs were initially processed at room temperature, but once the cells were incubated at 37°C with various reagents, they were then kept at 37°C with warm medium for the rest of the experiment. Such processing yielded ≥ 99% viable PMNs with no mononuclear cell contamination as determined by morphology with Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis for FcR III (CD16) that is expressed on freshly-isolated PMNs, but not on freshly-isolated monocytes and eosinophils,23 confirmed that the preparations usually contained >99% CD16+ cells.24 Dual staining of phycoerythrin-labeled CD16+ PMN preparations with fluorescein isothiocyanate–labeled anti-CD14,25 further indicated that monocytes were absent (data not shown).

Cytokines: and cell culture. All experiments performed in this study were performed with endotoxin-free media and supplies to avoid nonspecific activation of PMNs. Cells were cultured in RPMI 1640 media containing 5% heat-inactivated human AB serum (Flow Laboratories, McLean, VA), with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 mmol/L HEPEs buffer (GIBCO Laboratories, Grand Island, NY), and will subsequently be referred to as the complete medium. In some experiments, PMNs were incubated for 4 to 6 hours at 37°C in the presence or absence of 1,000 U/mL recombinant human IL-2 (2 x 10^5 U/mg, specific activity), 1,000 U/mL of GM-CSF (Immunex Corp, Seattle, WA), or 10 ng/mL of IL-8. These concentrations were previously determined to induce maximal activation of PMNs.27 28 All cytokines contained less than 0.1 ng/mL of endotoxin as determined by the Limulus amoebocyte lysate assay (MA Bioproducts, Walkersville, MD).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Southern Hybridization

Total RNA was extracted from freshly isolated PMNs or PBMCs as previously described.30 One microgram of total RNA was used as template for first-strand cDNA synthesis in a 20 µL reaction mixture containing 0.5 µg oligo (d)T primer (Promega, Madison, WI), 0.5 mmol/L each dATP, dCTP, dGTP, and dTTP (dNTP) and 200 U reverse transcriptase (GIBCO-BRL Life Technologies, Gaithersburg, MD). The first-strand cDNA was amplified by PCR using specific γc oligonucleotide primers synthesized on an PS 250 DNA synthesizer (Cruachem, Dulles, VA) based on published sequence data.30 Both oligonucleotide primers are specific for γc and were designed to amplify a 1,427-nucleotide fragment in both PMNs and PBMCs (data not shown). To control for the amplification of genomic DNA from PMNs and PBMCs, parallel experiments were performed without the addition of the RT and no bands were detected. To assess the specificity of the PCR reaction, the cDNA was then transferred to nitrocellulose and analyzed by Southern hybridization with a γc 32P-labeled cDNA probe. A single hybridization signal was shown for both PMNs and PBMCs (data not shown). The same RT product, amplified with oligonucleotide primers specific for β-actin and probed with a 32P-labeled β-actin cDNA probe, confirmed approximately equal amounts of intact mRNA present in the PBMC and PMN RNA samples.

Constitutive Expression of γc mRNA in Human PMNs

We next confirmed the PCR results of PMNs by Northern blot analysis. PMNs free of monocytes and lymphocytes, with <1% eosinophil contamination, as determined by morphology and FACS analysis, were lysed and total RNA extracted to be probed for hybridization with a specific cDNA for γc. Each PMN population of four normal donors expressed similar levels of γc mRNA in PMNs and PBMCs (Fig 1A).

Certain cytokine receptors may be positively regulated by the same or other cytokines. For example, IL-2 enhances IL-2R in lymphocytes and IFNγ induces IL-2R expression in monocytes.34 Thus, it was important to define whether

RESULTS AND DISCUSSION

Identification of γc Gene by RT-PCR

Oligonucleotide primers derived from the published γc sequence31,32 were used for RT-PCR to detect γc gene expression in PMNs. Total RNA was prepared from freshly isolated PMNs of three normal blood donors. The mRNA expression of γc in PBMCs was used as a positive control. The corresponding mRNA for γc was detected as a 1,427-nucleotide fragment in both PMNs and PBMCs (data not shown). To control for the amplification of genomic DNA from PMNs and PBMCs, parallel experiments were performed without the addition of the RT and no bands were detected. To assess the specificity of the PCR reaction, the cDNA was then transferred to nitrocellulose and analyzed by Southern hybridization with a γc 32P-labeled cDNA probe. A single hybridization signal was shown for both PMNs and PBMCs (data not shown). The same RT product, amplified with oligonucleotide primers specific for β-actin and probed with a 32P-labeled β-actin cDNA probe, confirmed approximately equal amounts of intact mRNA present in the PBMC and PMN RNA samples.

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expression of the \( \gamma_c \) on PMNs can be regulated by cytokines. It has been reported that the 1.8-kb message of \( \gamma_c \) is dominant in human PBMCs. However, after stimulation with phytohemagglutinin (PHA), a second \( \gamma_c \) mRNA species of 3.6-kb was also observed in human PBMCs.\(^{31} \) Stimulatory agents, such as GM-CSF, IL-8, and IL-2 are known to activate PMN functions.\(^{3,7,24,27,28} \) To determine if PMNs from normal donors could respond to these three stimuli for induction of \( \gamma_c \) gene expression, PMNs were cultured for 6 hours at 37°C with either medium or 1,000 U/mL of GM-CSF or IL-2, or 10 ng/mL of IL-8. Then the total cellular RNA was extracted from each treated group. A representative experiment is shown in Fig 1B. All these stimulatory agents were not capable of enhancing the level of expression of the \( \gamma_c \) gene by Northern blot analysis, although the same cytokines could readily induce TNF\( \alpha \) gene expression in PMNs. These results showed the apparent constitutive expression of the \( \gamma_c \) chain gene in human PMNs that is consistent with the previous observations in lymphoid cells.\(^{31,32} \) It has been observed recently that the \( \gamma_c \) promoter lacks classic TATA motifs at typical distances relative to the transcription initiation sites and lacks kB and CArG motifs found in IL-2R\( \alpha \), which are required for the inducibility of this gene.\(^{32} \) These studies indicated that the \( \gamma_c \) chain is more likely to be constitutively expressed in lymphoid cell lines and has low level of inducibility after mitogenic stimulation.\(^{32} \) Our kinetic studies show that the expression of \( \gamma_c \) is not time-dependent because prolonged incubation with recombinant IL-2 could not increase \( \gamma_c \) mRNA expression in PMNs (Fig 2).

\( \gamma_c \) Protein Expression in Human PMNs

We next used a \( \gamma_c \)-specific rabbit polyclonal antibody directed against the cytoplasmic domain of the \( \gamma \)-chain for Western blotting. Both PBMC and PMN lysates were run on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose, and the membrane was immunoblotted with anti-\( \gamma_c \) antibody, or with preimmune rabbit serum as a negative antibody control. As shown in Figs 3 and 4, \( \gamma_c \) antibody in human PMNs identified three bands of \(-64, -58, \) and \(-50 \) kD from each donor, and the first two bands were also found in control PBMCs from two donors. Immunoblotting with preimmune rabbit serum instead of anti-\( \gamma_c \) antibody in both PMNs and PBMCs did not display any detectable proteins (data not shown) indicating that the bands detected with the anti-\( \gamma_c \) antibody were specific. It should be noted that the p64, p58, and p50 forms were always present in all PMNs, but their comparative levels may vary from donor to donor.

Because the whole cell lysates were subjected to Western blot analysis, both cell surface and intracellular forms of \( \gamma_c \) can be detected. The presence of additional bands detected by Western blot analysis most likely represented precursor forms. To confirm this possibility, the PMNs and PBMCs were treated with tunicamycin, which inhibits N-linked carbohydrate addition to proteins. As shown in Fig 4, control PMNs and PBMCs displayed three or two bands, respectively, as in Fig 3 (lanes 1 and 6). After exposure of PMNs and PBMCs to 10 \( \mu \)g/mL and 20 \( \mu \)g/mL of tunicamycin, respectively, for 12 hours, only a 39-kD band was detected (lanes 5 and 9). The 39-kD protein band is of a size equal to that of the predicted amino acid sequence of the cloned cDNA. The response of PMNs or PBMCs to tunicamycin was dose dependent, as shown in lanes 2 through 5 or lanes 7 through 9. In PBMCs, 10 \( \mu \)g/mL of tunicamycin produced an extra detectable band at 50 kD (lane 8), which is an intermediate glycosylated band normally present in PMNs before tunicamycin (lane 1). This result suggests that the \( \gamma_c \)
in PBMCs are usually glycosylated as p64 and p58 forms, and incomplete glycosylation at 10 μg/mL of tunicamycin could then show smaller forms of the γ chain, i.e., p50 and p39. In PMNs, concentrations of tunicamycin below 10 μg/mL only partially prevented the glycosylation of newly synthesized proteins. There was no significant difference in viability or survival between tunicamycin-untreated and tunicamycin-treated PMNs at all doses tested (data not shown).

Numerous cell types, including T, B, natural killer (NK), and monocytes, constitutively express IL-2Rβ and require activation to express IL-2Rα. The association of IL-2Rα with IL-2Rβ/γ chain forms the high-affinity IL-2R. Our previous studies have shown that PMNs constitutively express IL-2Rβ, but not IL-2Rα. The affinity of IL-2 binding to PMNs were found to fit a single site model and indicated that the IL-2R on PMNs bound to IL-2 with intermediate affinity; kD, 1.1 × 10^9 mol/L. This approximated the affinity of IL-2R found on the NK cell–like YT cell line. However, it has been reported that the IL-2 binding sites created by expressing the recombinant β chain in fibroblast cells were of a very low affinity, in sharp contrast with intermediate-affinity binding usually found on lymphoid cells. Because both β and γ chains were required to obtain intermediate-affinity binding, we hypothesized that γ chain must also be expressed on PMNs. Using RT-PCR, Northern blot analysis, and Western blot analysis, we found that freshly isolated human PMNs constitutively express γ chain at both mRNA and protein levels. Three forms of the γ chain were shown on human...
PMNs by Western blot analysis that were p64, p58, and p50. Normal human PBMCs, in comparison, showed only the p64 and p58 bands. After treatment with tunicamycin, only a single 39-kD band was detected in both PMNs and PBMCs. These data indicate that translation of a single γc protein occurred that was later processed by glycosylation to yield various-sized γc forms. Interestingly, the known PMN activating cytokines, GM-CSF, IL-8, and IL-2, were unable to enhance γc gene expression, indicating that γc is constitutively expressed on PMNs.

Experimental evidence has suggested that the γc plays a pivotal role in facilitating IL-2 binding by IL-2Rβ and in receptor signaling. Recent studies have shown the existence of a stable IL-2–IL-2R γc complex. Thus, these studies establish that the γc chain directly contributes to the IL-2 binding site, consistent with the hypothesis that γc chain influences IL-2R affinity through its direct interaction with IL-2. What is most important is our finding that γc gene expression in PMNs is at a remarkably high level, equivalent to that seen in PBMCs. It is tempting to speculate that the γc chain participates in IL-2 function and PMN functions will be the subject for future studies. This pursuit is of great importance because it will yield insight into the normal biologic role of γc in clinically important cells, such as PMNs.

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