Expression of Interleukin-2 Receptor γ Chain on Human Neutrophils

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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, γ_c. The present study was undertaken to determine whether human polymorphonuclear neutrophils (PMNs) expressed γ_c chain. Reverse transcription-polymerase chain reaction and Northern blot analysis showed that fresh human PMN constitutively expressed a remarkable level of γ_c 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 γ_c gene expression. Western blot analysis with a rabbit anti-γ_c polyclonal antibody identified 64-, 58-, and 50-kD γ_c bands in lysates from PMN, but only 64- and 58-kD 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 γ_c and the three forms detected are caused by different glycosylation of a protein translated from a single mRNA species.

INTERLEUKIN-2 (IL-2) plays a central role in the clonal expansion of activated T-cells with specific surface receptors.[1,2] The IL-2/IL-2 receptor (IL-2R) system has been widely studied in lymphocytes,[2] but it is becoming apparent that IL-2 can mediate multiple biologic functions in nonlymphoid cells, including activation of monocytes and granulocytes.[3-7] These observations suggest that IL-2 is a principal regulator among immune cells and IL-2 delivers various signals to a wide range of cell types via interaction with its cell surface receptor. The IL-2R is unique among growth factor receptors in that it is made up of at least three distinct membrane components: IL-2Rα, IL-2Rβ, and γ_c, which is a newly identified peptide chain unrelated to any known molecule including the FcRγ. Different combinations of these three distinct chains dictate the affinity of the IL-2R. Whereas low-affinity receptors contain IL-2Rα chain, intermediate-affinity IL-2R contain IL-2Rβ and γ_c chains. High-affinity receptors contain all three chains.[1,2] γ_c is encoded by the gene that is defective in X-linked severe combined immunodeficiency[8] and has also been shown to be a functional component of IL-4 and IL-7 receptors.[9-12]

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.[13] 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 (IFNγ), and granulocyte macrophage-colony stimulating factor (GM-CSF).[13,14] 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.[15,16] 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.[17] 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.[18] We have shown that PMNs can respond to IL-2 with increased antifungal activity, prolonged survival in vitro, and increased IL-8 and TNFα-induced gene expression.[3,7,19,20] Circulating human PMNs express intermediate-affinity receptors for IL-2 as measured by Scratchard analysis and express IL-2Rβ, but not IL-2Rα.[3]

The discovery of the intermediate-affinity receptor on PMNs and knowledge that heterodimerization of IL-2Rβ and γ_c is required for signaling[21] suggested to us that γ_c may likely be expressed on PMNs. Therefore, we wished to explore the possibility that γ_c 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 γ_c mRNA and protein, and that the cytokines known to activate PMN function did not alter γ_c 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 400*g* 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 200*g*, 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 eosinophil[22,23] confirmed that the preparations usually contained >99% $CD16^+$ cells.[24] Dual staining of phycoerythrin-labeled $CD16^+$ PMN preparations with fluorescein isothiocyanate–labeled anti-CD14[25,26] 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 (2×10^7 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.[27-29] 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 20μL reaction mixture containing 0.5 μg oligo (dT) primer (Promega, Madison, WI), 0.5 mmol/L each deoxynucleotide triphosphate (dNTP) and 200 U reverse transcriptase (GIBCO-BRL Life Technologies, Inc, Gaithersburg, MD). The first-strand cDNA was amplified by PCR using specific γ_c oligonucleotide primers synthesized on an PS 250 DNA synthesizer (Cruachem, Dullea, VA) based on published sequence data.[30] Both oligonucleotide primers are given from 5′ to 3′. γ_c (+) strand primer is 5′-GAA GAG CAA GCG CCA TGT TGA AGC C-3′; γ_c (-) strand primer is 5′-TTC TCA TCG GTT CAG GAA CAA TCG G-3′.

PCR reactions were performed using Vent Polymerase (New England Biolabs, Beverly, MA), and 25 cycles of 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 30 seconds. Then the reaction was extended for 5 minutes at 72°C. The RT-PCR product of γ_c was fractionated in a 1% agarose gel, stained with 2 μg/mL of ethidium bromide and transferred to Nytran filters (Schleicher & Schuell, Keene, NH). The filter was hybridized with a deoxycytidine triphosphate (dCTP)-^{32}P (Amersham Life Sciences, Arlington Heights, IL) random-primed-labeled cDNA probe for γ_c (a generous gift from K. Sugamura, Tohoku University School of Medicine, Sendai, Japan). After extensive washing, the blot was exposed to Kodak XAR film (Eastman-Kodak, Rochester, NY).

Isolation of total cellular RNA and Northern blot analysis. Total cellular RNA from 5×10^7 PMNs treated with medium or cytokines.[30] Each sample of RNA was denatured in a glyoxal-dimethyl sulfoxide mixture, fractionated on a 0.8% agarose gel, transferred to Nytran filters, and stained with methylene blue acetate to determine the presence and integrity of transferred RNA. Prehybridization was performed at 45°C in a 50% deionized formamide solution. Hybridization was at the same temperature for 18 hours with the ^{32}P-labeled γ_c cDNA probe. After hybridization, blots were washed at 60°C with 1 × SSC, 0.2% SDS, and 1 mmol/L EDTA and autoradiographed.

Antibodies. A rabbit polyclonal antibody to the intracytoplasmic portion of the γ_c was generated as previously described.[10,11]

Western blot. For Western blotting, fresh PMNs and PBMCs were solubilized by incubation at 4°C for 30 minutes in lysing buffer (0.5% NP-40, 10 mmol/L TRIS, 140 mmol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L iodoacetamide, 50 mmol/L NaF, 0.4 mmol/L Na orthovanadate, 1 μg/mL leupeptin, and 1 μg/mL aprotinin). In some experiments, cells were treated with various amounts of tunicamycin to prevent glycosylation of newly synthesized proteins before Western blot analysis. Then, 100 μg of whole cell lysates from PMNs and 20 μg from PBMCs were separated on a 7.5% SDS-polyacrylamide gel under reducing conditions, and transferred to Immobilon membranes. After blocking with 5% milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20, the membranes were immunoblotted with anti-γ_c or preimmune serum for 2 hours at room temperature and washed with PBS with 2.5% milk, 0.2% Tween 20, incubated with ^{125}I-protein A (Amersham Life Sciences) at 0.2 μCi/mL for 2 hours, and autoradioigraphed.

RESULTS AND DISCUSSION

Identification of γ_c Gene by RT-PCR

Oligonucleotide primers derived from the published γ_c sequence[31,32] were used for RT-PCR to detect γ_c gene expression in PMNs. Total RNA was prepared from freshly isolated PMNs of three normal blood donors. The mRNA expression of γ_c in PBMCs was used as a positive control. The corresponding mRNA for γ_c 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 γ_c ^{32}P-labeled cDNA probe. A single hybridization signal was shown for both PBMCs and PMNs (data not shown). The same RT product, amplified with oligonucleotide primers specific for β-actin and probed with a ^{32}P-labeled β actin cDNA probe, confirmed approximately equal amounts of intact mRNA present in the PBMC and PMN RNA samples.

Constitutive Expression of γ_c 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 γ_c. Each PMN population of four normal donors expressed similar levels of γ_c 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[33] and IFNγ induces IL-2R expression in monocytes.[34] Thus, it was important to define whether

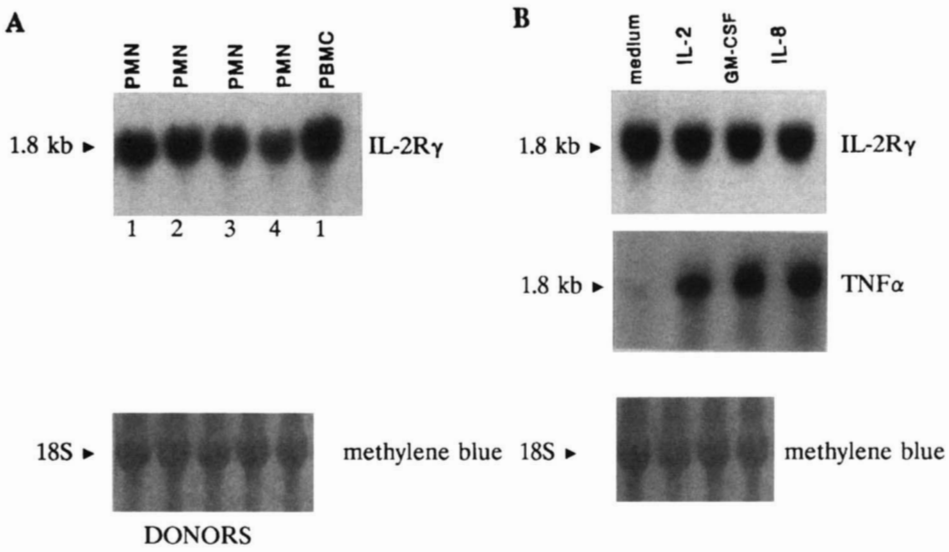

Fig 1. Northern blot analysis of IL-2R γ_c from human neutrophils. (A) shows IL-2Rγ mRNA expression from PMNs of different donors. Total RNA was isolated from fresh PMNs of four donors and fresh PBMCs of first donor. Twenty micrograms of total RNA was analyzed and hybridized with a ^{32}P-labeled human γ_c cDNA. The lower panel showed that the equal amounts of RNA were loaded per lane by methylene blue staining. (B) shows stimulation of neutrophils with IL-2, GM-CSF, and IL-8 for γ_c expression. Human PMNs were incubated with medium, IL-2, GM-CSF, and IL-8 for 6 hours. Total RNA was purified and Northern blot analysis for γ_c mRNA was performed as described in experimental procedures. After intensively washing, the same blot was rehybridized with human TNFα cDNA. These data were from a representative experiment undertaken on four separate donors.

expression of the γ_c on PMNs can be regulated by cytokines. It has been reported that the 1.8-kb message of γ_c is dominant in human PBMCs. However, after stimulation with phytohemagglutinin (PHA), a second γ_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 γ_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 γ_c gene by Northern blot analysis, although the same cytokines could readily induce TNFα gene expression in PMNs. These results showed the apparent constitutive expression of the γ chain gene in human PMNs that is consistent with the previous observations in lymphoid cells.[31,32] It has been observed recently that the γ_c promoter lacks classic TATA motifs at typical distances relative to the transcription initiation sites and lacks kB and CArG motifs found in IL-2Rα, which are required for the inducibility of this gene.[32] These studies indicated that the γ_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 γ_c is not time-dependent because prolonged incubation with recombinant IL-2 could not increase γ_c mRNA expression in PMNs (Fig 2).

γ_c Protein Expression in Human PMNs

We next used a γ_c-specific rabbit polyclonal antibody directed against the cytoplasmic domain of the γ-chain[19,20] 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-γ_c antibody, or with preimmune rabbit serum as a negative antibody control. As shown in Figs 3 and 4, the γ_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-γ_c in both PMNs and PBMCs did not display any detectable proteins (data not shown) indicating that the bands detected with the anti-γ_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 γ_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 μg/mL and 20 μg/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 μg/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 γ_c

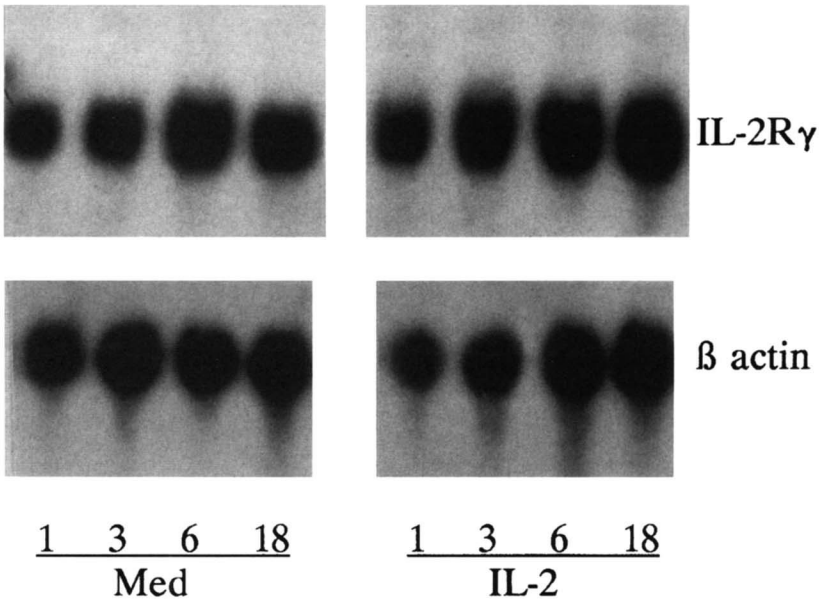

Fig 2. Kinetics of γ_c mRNA induction from medium or IL-2–treated PMNs. Human PMNs were treated with medium or IL-2 for the indicated times and then total RNA (20 μg) was purified. Northern blot analysis for γ_c mRNA was performed as described in experimental procedures. A representative experiment from four separate donors is shown.

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, ie, 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.[33,35-37] The association of IL-2Rα with IL-2Rβ/γ_c forms the high-affinity IL-2R.[31] Our previous studies have shown that PMNs constitutively express IL-2Rβ, but not IL-2Rα.[3] 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,[31] we hypothesized that γ_c 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_c at both mRNA and protein levels. Three forms of the γ_c were shown on human

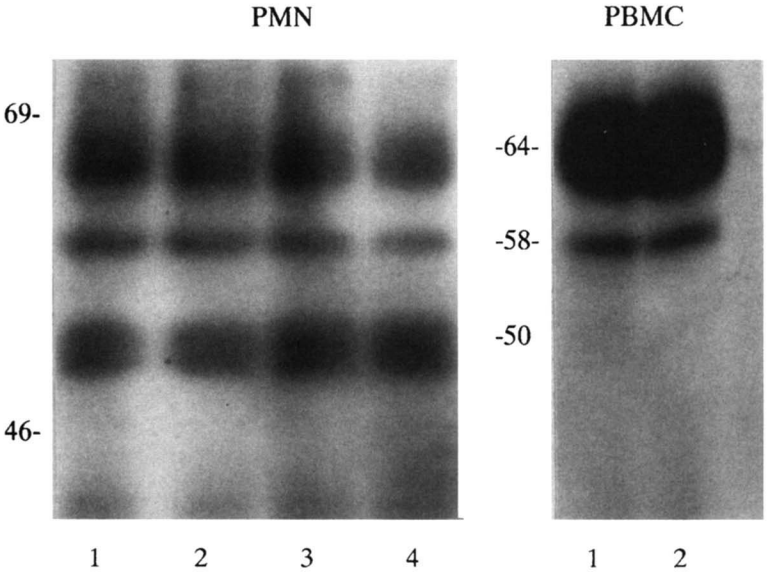

Fig 3. Detection of γ_c protein from human PMNs by Western blot analysis. Molecular weight was indicated in kilodaltons at left. Whole cell lysates were obtained from fresh PMNs of four donors and from fresh PBMCs of first two donors. One hundred micrograms of total proteins from PMNs and one fifth as much sample from the PBMCs were loaded onto a 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to a nitrocellulose filter. The filter was incubated with 1:1,000 dilution of polyclonal anti-γ_c antibody.

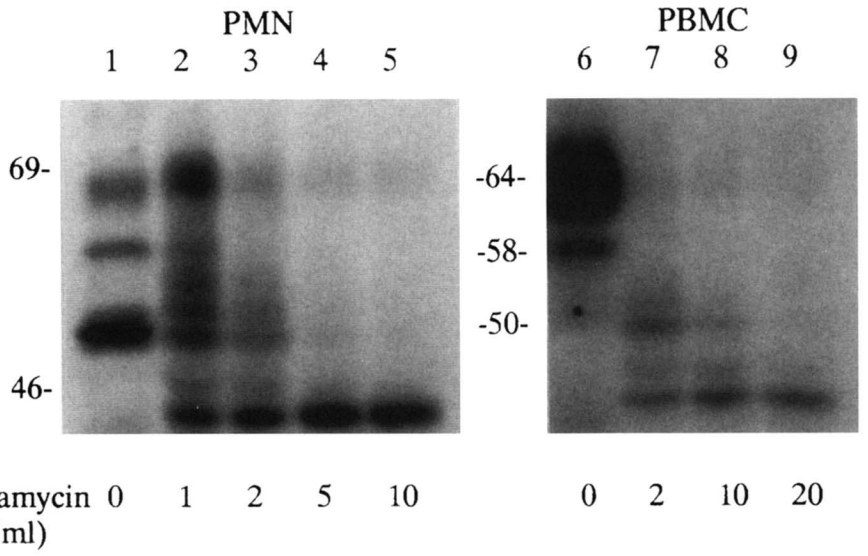

Fig 4. Effect of tunicamycin on γ_c glycosylation from human PMNs and PBMCs. Human PMNs and PBMCs were incubated with various amounts of tunicamycin for 12 hours before Western blot analysis. These data were from a representative experiment of four different donors.

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 γ_c protein occurred that was later processed by glycosylation to yield various-sized γ_c forms. Interestingly, the known PMN activating cytokines, GM-CSF, IL-8, and IL-2, were unable to enhance γ_c gene expression, indicating that γ_c is constitutively expressed on PMNs.

Experimental evidence has suggested that the γ_c plays a pivotal role in facilitating IL-2 binding by IL-2Rβ and in receptor signaling.[1,2,31] Recent studies have shown the existence of a stable IL-2–IL-2R γ_c complex.[38] Thus, these studies establish that the γ_c chain directly contributes to the IL-2 binding site, consistent with the hypothesis that γ_c chain influences IL-2R affinity through its direct interaction with IL-2. What is most important is our finding that γ_c gene expression in PMNs is at a remarkably high level, equivalent to that seen in PBMCs. It is tempting to speculate that the γ chain also forms a functional component of other cytokine receptors in PMNs, as has been shown in lymphocytes or IL-4R– and IL-7R–transfected COS cells.[9-12] The possibility of the use of the γ_c chain in other receptors important in 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 γ_c in clinically important cells, such as PMNs.

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