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Expression of functional receptors for interleukin-6 by human polymorphonuclear leukocytes

Downregulation by granulocyte-macrophage colony-stimulating factor

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Surface interleukin-6 receptors were identified on human polymorphonuclear leukocytes (PMNL) by monoclonal anti p80-chain antibody MT 18. Cytoplasmic RNA harvested from PMNL also contained IL-6-R transcripts. Binding of recombinant human (rh) interleukin-6 (IL-6) to IL-6-R bearing PMNL was identified by flow cytometry using phycoerythrin (PE)-conjugated ligand. Treatment of PMNL with rh granulocyte-macrophage colony-stimulating factor (GM-CSF) led to the inability of PMNL to bind MT 18 monoclonal antibody (moAb) and to display binding sites for PE-conjugated rh IL-6. Levels of IL-6-R transcripts in PMNL exposed to GM-CSF were about 5-fold below those of PMNL cultured in medium only. Though a definitive role for IL-6 to modulate the function of PMNL was not found, treatment of PMNL with rh IL-6 clearly resulted in an enhancement of transcript levels of the early response genes *c-fos* and *c-jun* in these cells, thus indicating that IL-6 binding is followed by signal transduction.

Neutrophil; IL-6 receptor; GM-CSF; Receptor downregulation

1. INTRODUCTION

Interleukin-6 is one of the central mediators of the complex reaction of the host to injury or infection referred to as the acute phase response ([1,2] for review). Important sites of IL-6 synthesis include monocytes, blood vessel endothelials, smooth muscle cells, connective tissue fibroblasts, chondrocytes, osteoblasts, keratinocytes, mesangial cells, astrocytes, anterior pituitary cells, and stromal endometrium cells ([3] for review). We have recently shown that PMNL may also be able to produce IL-6, upon activation with GM-CSF or tumor necrosis factor [4]. The physiological effects exerted by IL-6 include induction of fever, induction of immunoglobulin synthesis in activated B-cells, activation of T-cells and natural killer cells, stimulation of megakaryopoiesis, induction of acute phase response synthesis by the liver, and corticotropin release by cells of the pituitary gland ([3] for review). IL-6 acts on its target cells via a specific dimeric receptor. A 80 kDa membrane protein serves as IL-6 binding site [5]. IL-6 binding is followed by association of the 80 kDa protein with a second 130 kDa transmembrane protein providing signal transduction [6]. Given the previous demonstration by several investigators, including ourselves, of biosynthetic activity of human PMNL upon exposure to cytokines [4,7–9], and the abundant presence of PMNL in many organs participating in the acute phase reaction, we thought to determine whether IL-6 was also able to act on PMNL. To that end, IL-6-R expression and IL-6 binding by PMNL was explored. A series of other cytokines involved in acute phase responses were also investigated to address their possible role in modulating IL-6 receptiveness of PMNL.

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2. MATERIALS AND METHODS

2.1. *Purification of PMNL*

PMNL were separated from heparinized peripheral blood obtained from consenting healthy volunteers by density separation using Ficoll-Hypaque (Pharmacia Laboratories, Uppsala, Sweden), followed by dextran sedimentation as previously described [4]. Culture of PMNL was performed in serum-free culture medium prepared as outlined below.

2.2. *Preparation of serum-free culture medium*

Serum-free culture medium was prepared from powdered Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY). To 900 ml of reconstituted IMDM triple-destilled water (40.5 ml), sodium bicarbonate (3.0 mg/ml), 3-mercapto-1,2-propanediol (8 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml; Sigma Chemicals, Munich, Germany) were added. Five ml of 10% fatty acid-free deionized bovine serum albumin was added to 44.35 ml of prepared IMDM. To this mixture was added 0.65 ml transferrin (90 mg/ml) saturated with 7.0 mmol/l ferric chloride in 1 mmol/l HCl (Sigma) [10].

2.3. *Monoclonal antibodies and cytokines*

MoAb MT18 detecting the p80 IL-6-R chain was obtained from T. Hirano (Biomedical Research Center, Osaka University, Osaka, Japan) [11]. Anti-CD-16 moAb were purchased from Becton Dickin-

son (Heidelberg, Germany), and included phycoerythrin-conjugated anti-Leu-11c (clone B 73.1) and fluoresceinated anti-Leu-11a (clone NKP 15). Phycoerythrin-conjugated rh IL-6 and rh IL-8 were purchased from British Bio-Technology (Cowley, Oxford, UK). Recombinant human IL-3 and GM-CSF were kindly provided by Immunex (Seattle, WA) and were obtained through D. Krumwieh (Behringwerke AG, Marburg, Germany). Recombinant human macrophage (M)-CSF and leukemia inhibitory factor (LIF) were gifts of M. Garnick and G. Wong (Genetics Institute, Cambridge, MA). Recombinant human granulocyte (G)-CSF was kindly donated by L. Souza (Amgen, Thousand Oaks, CA). Recombinant human IL-4 was a gift of P. Trotta (Schering Corp., Bloomfield, NJ).

2.4. *Immunofluorescence analysis*

10^6 PMNL/ml in serum-free culture medium were either cultured for the indicated period of time prior to immunofluorescence analysis or were directly subjected to flow cytometric two-color immunofluorescence using FACS Star flow cytometer [12] (Becton Dickinson). Staining with MT 18 moAb was done with 25 μg antibody/ml of phosphate-buffered saline (PBS; Gibco) for 30 min, that was followed by an incubation (30 min) with fluoresceinated goat anti-mouse IgG (Becton Dickinson) at 20 μg/ml. Incubation with anti-CD 16-PE and fluoresceinated anti-CD 16 (25 μg/ml) was also done for 30 min. Negative nonbinding control antibodies used at 850 μg/ml each included fluoresceinated – or PE – conjugated mouse IgG_1 or IgG_{2a+b} (Becton Dickinson). The concentration of IL-6-PE to be used for staining of 10^6 PMNL was 100 ng/ml. In experiments analyzing IL-6 binding, a 10× concentrated PBS was used for washing procedures to minimize background and enhance specific binding.

2.5. *Isolation of RNA, cDNA probes, RNA blotting*

Total RNA was extracted after lysis of cells in guanidinium isothiocyanate followed by separation on a cesium chloride gradient [13]. RNA was electrophoresed on denaturing formaldehyde gels and blotted onto nylon membranes (Nytran; Schleicher & Schuell, Dassel, Germany). Prehybridization was carried out at 42°C in the presence of 5 × SSC (1 × SSC = 0.15 mol/l NaCl, 0.015 mol/l sodium citrate), 50% formamide, 1% Na_2DoSH_4, 5 × Denhardt's solution and 100 μg/ml denatured sonicated salmon sperm DNA. Hybridization was performed using oligolabeled [14] cDNA probes. The IL-6R cDNA probe was a 1.7 kb *Hin*dIII/*Xba*I fragment purified from the pBSF2R.236 plasmid kindly provided by T. Hirano [5]. The 800-bp *Bam*HI/*Pst*I fragment of a human α-actin cDNA (pAC 289) was kindly provided by J. Ramadori (Department of Medicine, University of Mainz, Germany) [16]. The 1.2 kb *Eco*RI/*Bam*HI fragment [16] of human *c-jun* in p-Bluescript SK was provided by P. Angel (Dept of Pharmacology, University of California, San Diego, CA). The 900 bp *Nco*I/*Sca*I fragment of human *c-fos* in pBR322 was purchased from the American Tissue Type Culture Collection (Rockville, MD) [17]. Hybridization took place at 42°C in the presence of 2 × SSC, 50% formamide, 1% Na_2DoSH_4, 10% dextran sulfate, 5 × Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and probe labeled to high specific activity (1×10^6 cpm/ml hybridization mixture). Filters were washed in decreasing concentrations of SSC at 65°C to final stringency of 0.1 × SSC for 15 min and exposed to X-AR5 X-ray film (Kodak, Rochester, NY) at −70% using intensifying screens.

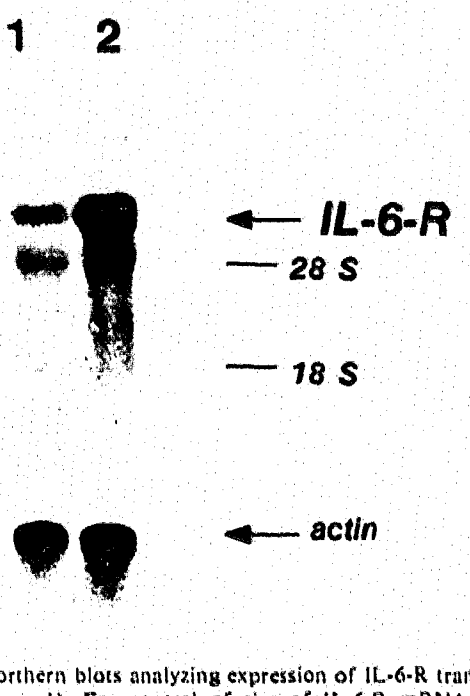

Fig. 2. Northern blots analyzing expression of IL-6-R transcripts in PMNL (lane 1). For control of size of IL-6-R mRNA (4.8 kb), cytoplasmic RNA obtained from blood monocytes were also hybridized with the IL-6-R specific cDNA (lane 2). Each lane contains 20 μg RNA. Experiments with PMNL from two different donors (not shown) gave identical results.

3. RESULTS

Purification of PMNL yielded preparations contain-

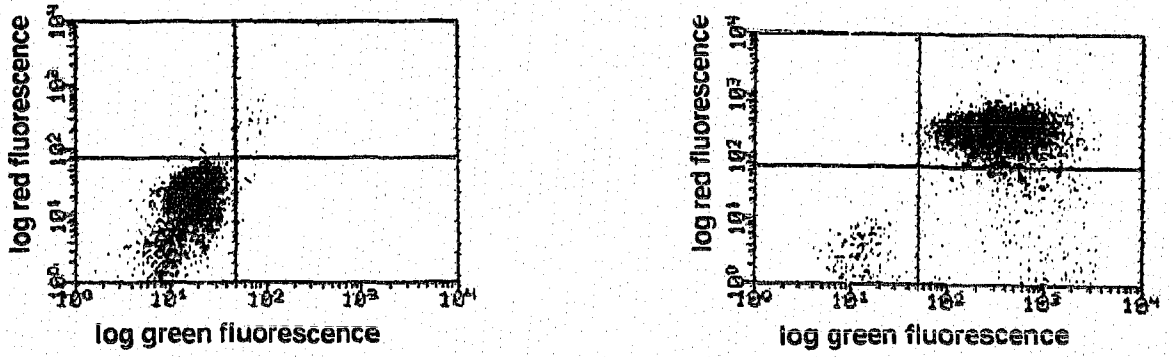

Fig. 1. Two-color fluorescence analysis of PMNL expressing CD16 (moAb anti-Leu-11c) and IL-6-R (moAb MT 18). The left panel represents negative controls using irrelevant non-binding PE-, or fluorescein-conjugated antibodies. The right panel shows staining of PMNL with IL-6-R moAb (log green fluorescence) and CD16-PE (log red fluorescence). The upper right chart of this panel represents PMNL that are double stained with both moAb. The data show a representative experiment. Three further experiments with PMNL from different donors gave identical results.

ing 88–92% neutrophils, 0–8% eosinophils, 0–3% basophils and 0–1% mostly granular lymphocytes by morphology. This cell population was analyzed for surface expression of the p80 chain of the IL-6-R using two-color fluorescence flow cytometry employing moAb anti-MT 18 and anti-CD 16-PE. As shown in Fig. 1, more than 92% of cells expressing CD 16-PE were also stained with anti-MT 18 suggesting IL-6-R expression by PMNL. To further assess binding sites for IL-6 on the surface of PMNL, cells were analyzed using PE-conjugated rh IL-6 and fluorescein-conjugated anti-CD-16 moAb, demonstrating that almost all CD 16 positive PMNL bound IL-6. Two-color immunofluorescence analysis with PE-conjugated IL-6 and fluoresceinated MT 18 moAb gave identical results (not shown). Northern blot analysis of cytoplasmic RNA obtained from freshly separated PMNL using an IL-6-R specific cDNA disclosed IL-6-R transcripts (Fig. 2). Evidence for receptiveness of PMNL for rh IL-6 was further substantiated by demonstrating that culture of PMNL for 4 h in the presence of rh IL-6 (20 ng/ml) was able to enhance transcript levels of the early response genes *c-fos* and *c-jun* known to form the transcription factor AP-1 (Fig. 3), but had no effect on mRNA accumulation of the IL-6-R in cultures performed over a period of 24 h (Fig. 4). Levels of IL-6-R transcripts were also monitored upon exposure of PMNL (12–24 h) to other cytokines including GM-CSF (20 ng/ml), G-CSF (20 ng/ml), M-CSF (1000 U/ml), IL-3 (20 ng/ml), IL-4 (50 ng/ml), LIF (100 U/ml), and IL-8 (50 ng/ml). As shown in Fig. 4, treatment of PMNL with GM-CSF, but not with the other cytokines, led to decrease of IL-6-R mRNA. Densitometric analysis of autoradiograms after normalization for α-actin transcripts showed that GM-CSF mediated decrease of IL-6-R mRNA was 5-fold (not shown). Given this observation, we next examined the effects of GM-CSF on binding of MT 18 moAb and PE-conjugated IL-6 to PMNL (Fig. 5). After treatment with rh GM-CSF for 2 h, PMNL failed to display detectable IL-6-R. Similarly, exposure of PMNL to rh IL-6 (20 ng/ml) for only 30 min led to an almost complete disappearance of binding sites for MT 18 antibody (Fig. 6), but had no effect on levels of IL-6-R mRNA (at 12–24 h; Fig. 4).

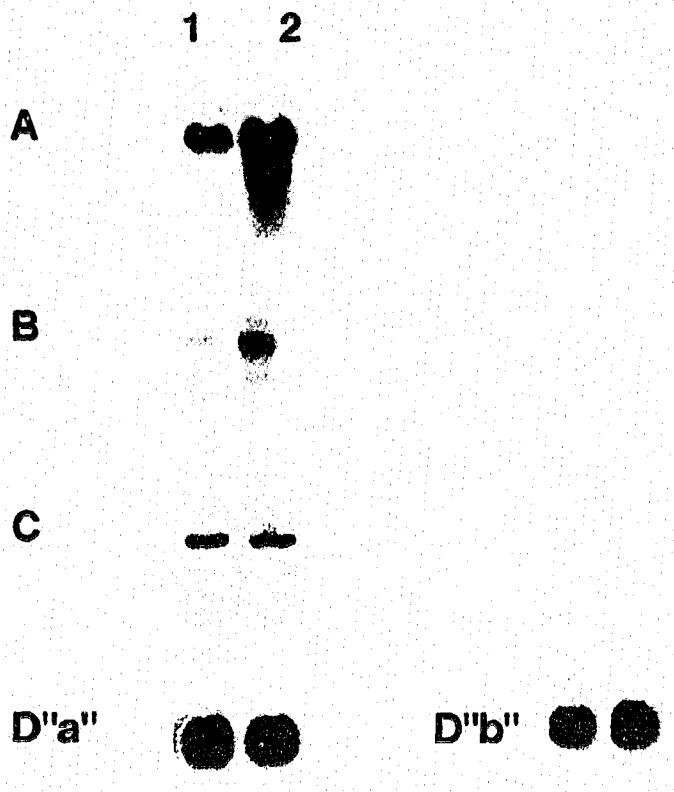

Fig. 3. Northern blots analyzing the effect of rh IL-6 treatment of PMNL on transcript levels of *c-fos* (panel A), *c-jun* (panel B) IL-6-R (panel C) and α-actin (panel D). PMNL were cultured in the presence (lane 2) or absence (medium control; lane 1) of rh IL-6 (20 ng/ml). Thereafter cytoplasmic RNA was collected and sequentially hybridized with specific cDNA probes. Culture time was either 4 h (for detection of *c-fos* and *c-jun* transcripts) or 12 h (for detection of IL-6-R mRNA). 20 μg RNA was loaded in each lane. α-Actin hybridization controls comparable RNA loading in single lanes. Lane 'a' represents actin levels after 4 h of culture, lane 'b' shows actin levels obtained after 12 h of cultures. Experiments with PMNL from two different donors gave identical results (not shown).

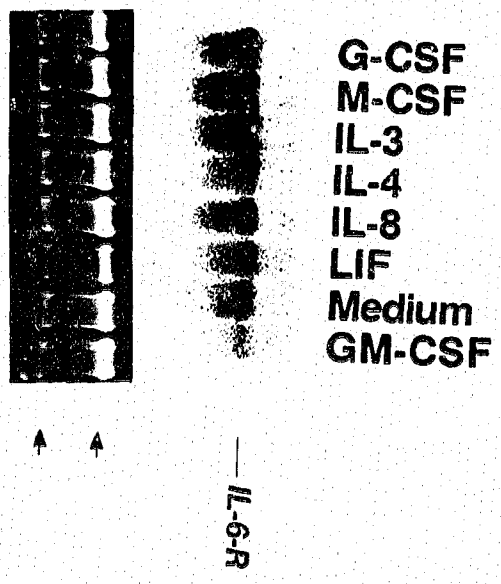

Fig. 4. Northern blots analyzing the effect of various cytokines on IL-6-R transcript levels. PMNL were exposed to various cytokines as indicated, or were exposed to medium only. After 12 h of culture cytoplasmic RNA was collected and subjected to analysis for IL-6-R mRNA. Cultures were also performed over a period of 24 h and gave comparable results (not shown). Integrity of RNA and identical RNA loading in single lanes was confirmed by ethidium bromide staining of ribosomal (r) RNA. Arrows indicate 28 S and 18 S rRNA. Experiments with PMNL from another donor gave identical results (not shown).

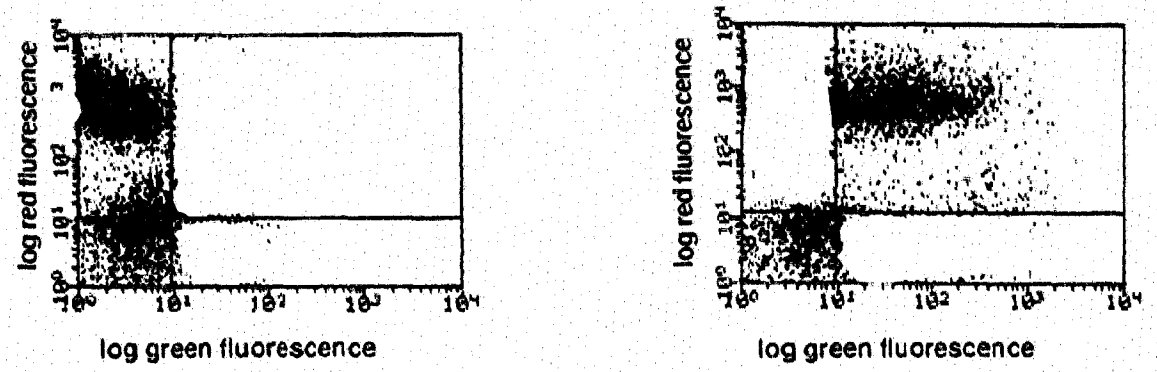

Fig. 5. Effect of rh GM-CSF treatment (2 h) on IL-6-R expression (moAb MT 18) by CD16 positive PMNL. The left panel shows two-color fluorescence analysis of PMNL with MT 18 moAb (log green fluorescence) and anti-CD16-PE (log red fluorescence) after culture in the presence of rh GM-CSF. In the right panel the same analysis was performed with PMNL exposed for a period of 2 h to medium only. The effect of exposure of PMNL to GM-CSF was also analyzed after 30 min and 60 min of culture, but failed to reveal changes in MT 18 surface expression (not shown). The data show a representative experiment. Three further experiments with PMNL from different donors gave identical results (not shown).

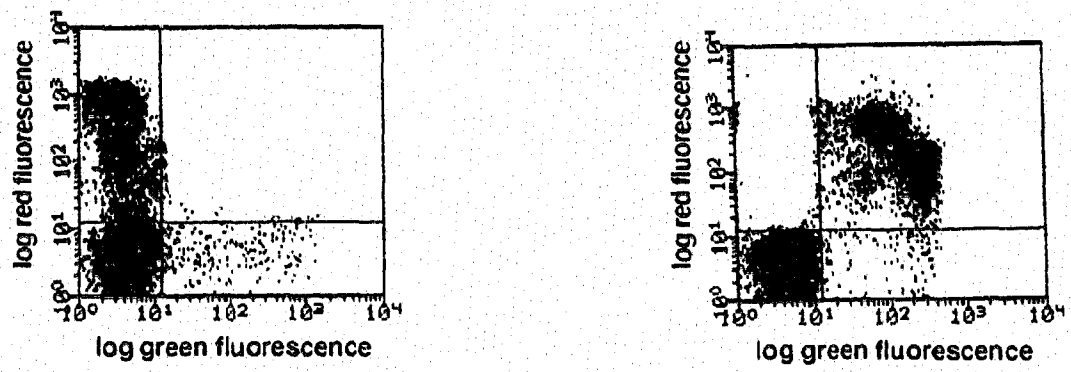

Fig. 6. Effect of rh IL-6 treatment (30 min) on IL-6-R expression (moAb MT 18) by CD16 positive PMNL. For protocol see legend to Fig. 5. Experiments with PMNL from another donor gave identical results (not shown).

4. DISCUSSION

We describe the presence of functional receptors for IL-6 on the surface of human polymorphonuclear leukocytes. PMNL exhibit levels of IL-6-R mRNA detectable by Northern blot analysis of cytoplasmic RNA, express surface binding sites for monoclonal antibody MT 18, known to identify the signal transducing p80 chain of the IL-6-R [19], and bind recombinant human IL-6. Exposure of PMNL to IL-6 results in increases in levels of transcripts of the early response genes *c-fos* and *c-jun*, forming the transcription factor AP-1. AP-1 binding sites are present in promoter/enhancer sequences of many regulatory genes [18]. There is thus indirect evidence to assume a fundamental role for IL-6 in PMNL function. In this regard we have analyzed the effects of IL-6 on superoxide anion production and the migration behavior of PMNL (data not shown), but failed to detect changes in both chemiluminescence and Boyldon chamber assays. Unlike in monocytes where IL-6 causes downregulation of IL-6-R mRNA [19], IL-6 treatment of PMNL failed to alter IL-6-R transcript levels. Exposure of PMNL to rh GM-CSF, however, led to decreased levels of stable IL-6-R transcripts, that was not observed when the effects of other cytokines including G-CSF, M-CSF, IL-3, IL-4, IL-8, and LIF were investigated. Mechanisms of down-modulation of IL-6-R transcripts by GM-CSF are still unknown and need further investigation. Nevertheless, we also observed an almost complete disappearance of IL-6 surface binding sites on PMNL as early as 2 h after initiation of cultures with GM-CSF. It is presently unclear whether upon GM-CSF exposure surface IL-6-R will internalize or will be shedded into the external environment. In preliminary experiments, not shown here, we failed to detect adsorbence of IL-6 by culture supernatants of GM-CSF treated PMNL, thus suggesting that GM-CSF treatment may not have caused shedding of IL-6-R. On a speculative basis one might assume that secretion of IL-6 by GM-CSF treated PMNL may have caused IL-6-R down-modulation. Time kinetics by which IL-6-R down-modulation in GM-CSF stimulated PMNL was observed support this hypothesis. Whereas

in IL-6 treated cells IL-6-R disappeared already as early as 30 min after initiation of culture, GM-CSF treatment required at least 2 h to down-regulate IL-6 binding and IL-6-R surface expression. Implication of that interpretation would be that GM-CSF induces IL-6 autocrinism.

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