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Hematopoietic Differentiation Activity of a Recombinant Human Interleukin-6 (IL-6) Isoform Resulting from Alternatively Spliced Deletion of the Second Exon

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We have previously identified and cloned an alternatively spliced form of human interleukin-6 mRNA lacking exon II, which encodes amino acid residues known to be important in gp130-mediated signal transduction pathways. We expressed and purified the recombinant protein (rIL6-alt) resulting from this alternatively spliced mRNA and now report the initial characterization of its biologic activities with comparison to full length IL6 (rIL6-full). rIL6-alt was found to have 10^4 to 10^5 fold less activity in proliferation assays with 7TD1 murine plasmacytoma cells and did not competitively inhibit the stimulatory activity of rIL6-full. In addition, like rIL6-full, rIL6-alt had antiproliferative activity toward M1 murine myeloblast cells and was 10–200-fold less active than rIL6-full. In contrast, in assays with human HL60 promyelocytic leukemia cells, rIL6-alt had greater antiproliferative activity than rIL6-full and more strongly upregulated phagocytosis as well as surface expression of the differentiation antigen CD11b. rIL6-full and rIL6-alt upregulated the level of lysozyme mRNA in HL60 cells approximately equally. These findings suggest that IL6-alt, which lacks amino acid residues encoded by the second exon of the gene, is not a natural inhibitor of IL6-full but may be relatively tissue specific and may play a role in modulation of hematopoietic cell growth and differentiation. Am. J. Hematol. 61:169–177, 1999. © 1999 Wiley-Liss, Inc.

Key words: interleukin-6; differentiation; alternatively spliced

INTRODUCTION

Interleukin 6 (IL6) is a multifunctional cytokine which has been shown to be involved in the control of proliferation and terminal differentiation of B-cells [1]. In addition, IL6 plays an important role in T-cell proliferation [1,2], the regulation of acute phase response proteins by hepatocytes [3], and in the growth of hematopoietic stem cells [1,4]. A significant body of evidence supports IL6 as a growth factor in multiple myeloma [5] and this cytokine has also been associated with other disorders including atherosclerosis [6], autoimmune diseases [7,8], Alzheimer's disease [9], and Kaposi's sarcoma [10]. The interleukin-6 molecule consists of a single polypeptide chain of approximately 22,000 Da molecular mass [1]. A dimer form of IL6 initially binds to a specific cell receptor p80, which then as a complex interacts with gp130 for high-affinity binding and signal transduction [11]. In addition to IL6, gp130 is known to be employed by other cytokines in transducing intracellular signals, including IL11, LIF, oncostatin M, and ciliary neurotrophic factor [12].

Previously we have reported the detection of an alternatively spliced form of human IL6 (IL6-alt) in peripheral blood mononuclear cells by using reverse transcription polymerase chain reaction (RT/PCR) [13]. We have cloned and sequenced this mRNA and found that it lacks the second exon and consists of a transcript that fuses the first exon in frame with the third exon of IL6. Because

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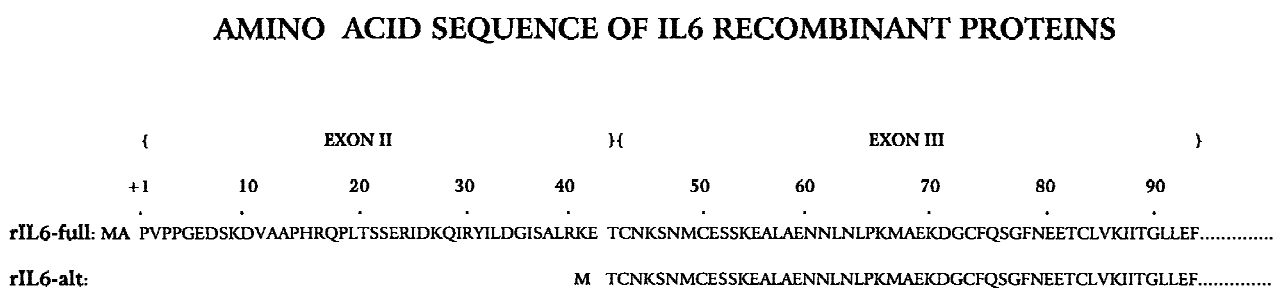

Fig. 1. Amino Acid Sequences of rIL6-full and rIL6-alt. In the rIL6-alt construct, amino acid residues +1-42 are deleted. Bold letters represent residues that are not found in the native IL6 sequence but have been added as part of the expression constructs. Only exons II and III are shown; exon IV encoding 49 amino acids and exon V encoding 55 amino acids (1) are identical in both rIL6-full and rIL6-alt.

IL6-alt lacks the entire exon II, which is known to encode a polypeptide region that interacts with the gp130 signal transduction domain receptor complex, we postulated that IL6-alt might represent a natural inhibitor of IL6. Alternatively, IL6-alt might have tissue-specific effects distinct from full-length IL6 (IL6-full).

In the mature secreted IL6-full molecule a 28 amino acid signal peptide consisting of the first exon and 22 amino acids of the second exon has been cleaved [1]. Other investigators have shown that constructs which delete amino acids +1 to +28 encoded in exon II of mature IL6 retain a high degree of bioactivity in a variety of assays [14]. In order to evaluate possible polypeptide forms of alternatively spliced IL6, we have constructed a recombinant form of IL6-alt which does not incorporate the first or second exons but starts at the proposed exon I-exon III splice site codon [13], with the addition of one ATG (methionine) start codon (Fig. 1). This IL6-alt would thus lack amino acids 1-42 found in IL6-full. In this report, we characterize the bioactivity of this recombinant IL6-alt polypeptide in comparison to rIL6-full.

MATERIALS AND METHODS

Construction of rIL6 Expression Clones

The recombinant human IL6 expression clone pT7.7/huIL6 [15] was obtained from the American Type Culture Collection (Rockville, MD). The alternatively spliced human IL6 (IL6-alt) expression clone was generated from the rIL-alt clone SS-1 previously described [13]. By using PCR, a modified 29-mer forward primer (5′-TC ATG ACA TGT AAC AAG AGT AAC ATG TGT) was employed to amplify rIL6-alt containing a *Bsp*HI (*Nco*I compatable) site with an ATG start codon proximal to the threonine codon at amino acid position +43 (Fig. 1). The reverse primer (5′-CTA CAT TTG CCG AAG AGC CCT CAG GCT GGA C) contained the 3′ end of the IL6 coding region ending on the termination codon. PCR reactions contained 20 μg/ml SS-1 clone DNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.25 mM $MgCl_2$, 250 μM dNTPs, 0.8 μM forward and reverse primers, and 60 U/ml Taq polymerase (Life Technologies, Bethesda, MD) in a final reaction volume of 50 μl. PCR reactions were run for 25 cycles of 1 min denaturation at 90°C, 1 min primer annealing at 55°C, and 1 min DNA synthesis at 72°C, after which a final 15 min incubation at 72°C was performed. The rIL6-alt PCR product was electrophoresed on a 1.5% agarose gel, excised and purified onto silica particles by using a Gene-Clean kit (BIO 101, Vista, CA). The purified DNA fragment was cloned into the PCR3.1-Uni vector (Invitrogen, Carlsbad, CA), and subsequently digested with *Bsp*HI and *Bam*HI. This fragment was then ligated directionally into the pET-15b expression vector (Novagen, Madison, WI), which had been digested with *Bam*HI and *Nco*I in order to produce a site compatible with *Bsp*HI.

Expression and Purification of Recombinant IL6-full and IL6-alt

Two methods of purifying rIL6-full and rIL6-alt were used, one employing guanidine-HCl extraction of inclusion bodies [15], and the other employing detergent solublization without guanidine-HCl (see Results). Cultures of IL6 expression constructs were grown in transformed *Escherichia coli* DE3 cells to OD_{590} of 0.6, diluted 1:25 into 500 ml of Luria broth containing 70 μg/ml ampicillin, and grown to an OD_{590} of 0.5. The cells were then induced 3 hr with 1 mM IPTG, centrifuged, washed with PBS, and either stored at −80°C or directly lysed by sonication. Cells from 500 ml cultures were resuspended in 20 ml of sonication buffer (20 mM Tris-HCl, pH 9.0, 2.5 mM EDTA, 1.6 mM PMSF), and sonicated for 20 min (15 sec on followed by 30 sec off) on ice by using a Fisher Model 550 Sonicator (Fisher Scientific, Pittsburg, PA) at setting 5. The sonicated lysates were centrifuged for 40 min at 1,400 × *g*, and the supernatant was saved. The pellet was sonicated a second time and centifuged as above, again saving the supernatant. The remaining pellet was sonicated two more times with the addition of 0.05% deoxycholate and 0.05% Tween-20, layered over

a 20% sucrose shelf, and centrifuged 30 min at 1,400 × *g*. The pellet was dissolved in 50 ml of 6 M guanidine-HCl containing 50 mM β-mercaptoethanol and 0.05% Tween-20, and dialyzed overnight against 20 mM Tris-HCl (pH 9.0)-5% glycerol-0.05% deoxycholate-0.05% Tween. The dialyzed proteins were then applied to a 2 ml DEAE Sepharose CL-6B (Pharmacia, Piscataway, NJ) column and eluted with a 0–400 mM NaCl gradient in 20 mM Tris-HCl (pH 9.0). The eluted fractions were analyzed by SDS-PAGE and Western blots, and the fractions that contained rIL6-alt were pooled and dialyzed against 10 mM HEPES (pH 8.0).

In order to prepare recombinant proteins without use of denaturing guanidine-HCl, the soluble proteins from detergent sonication supernatants were analyzed by sodium dodecyl sulfate (SDS)-PAGE, and those containing rIL6 with highest purity (usually, the last several supernatants) were pooled. These pooled supernatants were submitted to DEAE Sepharose CL-6B chromatography as described above, eluted, and dialyzed against 10 mM HEPES (pH 8.0).

SDS-PAGE and Western Blot Analysis

Recombinant IL6 proteins were electrophoresed on 0.1% SDS, 12% PAGE Laemmli gels, and either stained with Commassie Blue or electrophoretically transferred onto nitrocellulose filters in Towbin's buffer for 2 hrs at 150 mA as described [15,16]. Filters were probed with 1:200 diluted rabbit anti-human rIL6 antiserum or normal rabbit serum for 90 min at room temperature, washed, incubated further (1 hr, room temperature) with 1:5000 diluted alkaline phosphatase conjugated goat anti-rabbit Fc-IgG (Promega, Madison, WI), washed, and developed by using the Protblot AP System (Promega) as described [13,16].

Cell Lines

M1 murine myeloblast cells [17] and HL-60 human promyelocyte leukemia cells [18] were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM l-glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin (F10 medium). The cells were cultured at a density range of 10^5–10^6 cells/ml at 37°C in a 95% air/5% CO_2 humidified atmosphere. 7TD1, an IL6 responsive murine plasmacytoma cell line [19] was maintained in F10 medium supplemented with 100 pg/ml rIL6.

Cell Proliferation Assays (^{3}H-Thymidine Incorporation)

Cells (10^4/well) were placed into 96-well microtiter plates (Corning, Corning, NY) in 0.1 ml of F10 medium with titered amounts of rIL6-full or rIL6-alt and cultured for 66 hours after which 0.5 μCi/well ^{3}H-thymidine (100 Ci/mmol; ICN, Irvine, CA) was added to each well. After a further 6 hr of incubation, cells were harvested onto glass fiber filters by using a Dynatech Minimash 2000 cell harvester (Dynatech, Alexandria, VA) and radioactivity measured by using an aqueous-based scintillation fluid (Ecolume, ICN). All determinations were done in triplicate with <10% variability.

HL-60 Cell Culture

HL-60 cells (10^6 cells/ml/well) were cultured in F10 medium in 240-well (16 mm diameter) tissue culture plates (Corning) in the presence of 1 μg/ml rIL6-full, 1 μg/ml rIL6-alt, 50 ng/ml phorbol myristate acetate (PMA), 1% DMSO, or F10 medium alone for 16 or 72 hr (see figure legends). The cells were then harvested by centrifugation at 250 × *g* for 10 min and assayed as described below.

Mo1 (Anti-CD11b) Flow Cytometry Analysis

HL-60 cells which were cultured for 72 hr as described above were washed and resuspended at 3×10^6 cells/ml in PBS containing 1% fetal bovine serum and 0.1% sodium azide (PBN buffer). Cells (10^6) were then incubated on ice for 1 hr in the presence of 1:50 Mo1 (anti-CD11b) monoclonal antibody (Coulter, Hialeah, FL) or 25 μg of control normal mouse IgG (Sigma, St. Louis, MO). Cells were centrifuged, washed twice with 500 μl of PBN, resuspended in 350 μl of PBN, and incubated for 1 hr on ice in the presence of 1:50 FITC-goat anti-mouse IgG (Biosource International, Camarillo, CA). The cells were then centrifuged, washed twice with PBN, fixed in 500 μl PBS/1% formaldehyde, and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Phagocytosis By HL-60 Cells

One million HL-60 cells were cultured 68 hr in 24-well (16 mm diameter) tissue culture plates (Corning) in the presence of F10 medium alone (control), 50 ng/ml phorbol myristate acetate, 1 μg/ml rIL6-full, or 1 μg/ml rIL6-alt. Latex 1 μ fluorescent beads (Duke Scientific, Palo Alto, CA) were then added (150 beads per cell) followed by a further 4 hr incubation. Cells were then removed by agitation with a Pasteur pipette and centrifuged through a 0.4 ml cushion of fetal bovine serum (250 × *g*, 10 min) in order to remove free beads. After aspiration of the supernatant, the cell pellet was suspended in 200 μl of F10 medium, and at least 200 cells were analyzed for bead content by microscopy. Cells with ≥ 2 intracellular beads were considered positive.

RNA Isolation and RT-PCR Analysis

Total cellular RNA was isolated from approximately 10^7 cultured HL-60 cells by using guanidinium isothiocyanate/acid phenol-chloroform [20], and precipitated twice with 2.5 volumes of absolute ethanol at −80°C for

1 hr. All solutions for RNA preparation and use were made in diethyl pyrocarbonate-treated water. Precipitates were washed three times with cold 75% ethanol, dried under vacuum, and dissolved in 1 mM dithiothreitol containing 1 U/μl RNasin ribonuclease inhibitor (Promega). Prior to cDNA synthesis, the RNA was incubated at 60°C for 2 min and then chilled on ice. cDNA synthesis reaction mixtures contained 200 μg/ml RNA, 500 μM dNTPs, 50 mM Tris-HCl (pH 8.2), 12 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.5 mM spermidine, 50 μg/ml oligo-dT (12-18 mer), and 800 U/ml AMV reverse transcriptase (Promega) in a final volume of 15 μl. The reaction mixtures were incubated for 90 min at 42°C, followed by 95°C for 5 min, and 37°C for 30 min in the presence of 20 μg/ml DNase-free RNase. A 5 μl aliquot of the cDNA product was then submitted to PCR amplification in the presence of 0.8 μM of each appropriate forward and reverse primer, 1.25 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 20 μg/ml gelatin, 200 μM dNTPs, and 0.5 U Taq DNA polymerase in a final volume of 20 μl. Amplification reactions consisted of 25 cycles of 94°C denaturation (1 min), 55°C annealing (1 min) and 72°C synthesis (1 min), with a final extension at 72°C for 15 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels in 40 mM Tris-acetate (pH 8.5)-2 mM EDTA. *Hae*III-digested ϕX174 replicative-form DNA fragments were used as molecular weight markers. β-actin and lysozyme oligonucleotide primers used in this study have been previously reported by others [13,21].

RESULTS

SDS-PAGE and Western Blot Analysis of Purified rIL6-full and rIL6-alt

Purified rIL6-full and rIL6-alt were examined by SDS-PAGE and Western blot analysis as shown in Figure 2. rIL6-full and rIL6-alt migrated as bands of Mr 22 kd and 14 kd, respectively, and were reactive with anti-IL6 antibodies but not normal sera. Purification of rIL6-alt by guanidine-HCl extraction of inclusion bodies followed by renaturation resulted in reduced yields compared to rIL6-full (data not shown). This suggested that the renaturation/refolding process for rIL6-alt was less efficient.

Effect of rIL6-alt on Murine Plasmacytoma 7TD1 Proliferation

Figure 3 compares the effects of rIL6-full and rIL6-alt on proliferation of the IL6 responsive 7TD1 murine plasmacytoma line. In 5 experiments, the range of rIL6 necessary for 50% maximal stimulation of 7TD1 cells was 1–25 pg/ml and for rIL6-alt was 7–135 ng/ml. A commercial source of rIL6 induced 50% maximal stimulation at 50–100 pg/ml. Figure 3 depicts a representative experiment comparing rIL6-full and rIL6-alt activities in this assay and shows that rIL6-full was 10^4 to 10^5 times more active than rIL6-alt. Both guanidine denatured/ renatured and non-denatured preparations of rIL6 had similar activities. This strongly suggested that rIL6-alt had little B cell proliferative activity.

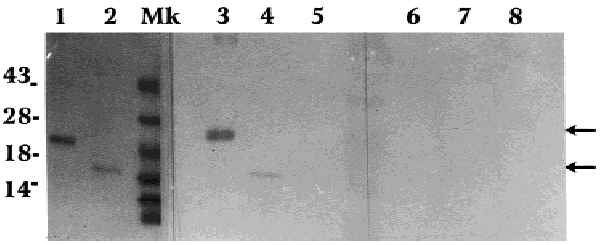

Fig. 2. SDS-12% PAGE and Western Blot Analysis of Purified rIL6-full and rIL6-alt. Recombinant proteins were purified as described in Materials and Methods and submitted to SDS-PAGE and Western analysis with anti-IL6 antibodies. The lanes are: 1, 3, and 6, rIL6-full (1 μg); 2, 4, and 7, rIL6-alt (0.3 μg); 5 and 8, control bacterial lysate from IPTG-induced *E. coli* DE3 transformed with pET-15b vector (without insert) representing 0.2 ml of culture at OD_{590} 0.5. Lanes 1-2 are from a Coomassie-stained SDS-PAGE gel, and lanes 3-8 are from a parallel Western blot probed with rabbit anti-IL6 (lanes 3-5) or normal rabbit serum control (lanes 6-8). Molecular weight markers (Mk) in kilodaltons (kd) are indicated at the left margin. The arrows along the right margin indicate the bands produced by rIL6-full (22 kd) and rIL6-alt (14 kd).

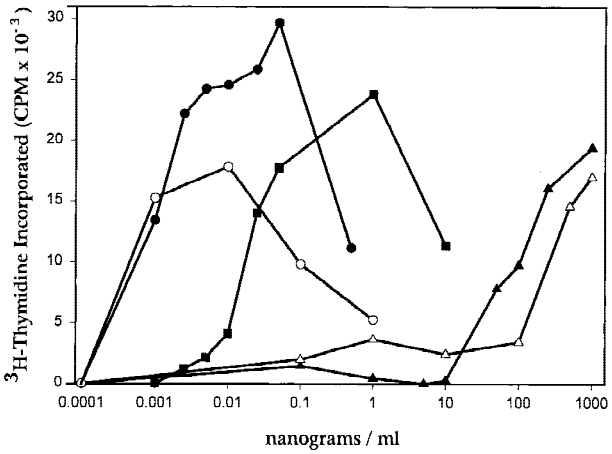

Fig. 3. Proliferative Response of 7TD1 Murine Plasmacytoma Cells to rIL6-full and rIL6-alt. Cells were cultured in 96-well plates at 10^4 cells/well for 66 hr in the presence of recombinant proteins followed by 6 hr culture with ^{3}H-thymidine and assay of incorporation into DNA. rIL6-alt (triangles) and rIL6-full (circles) were purified using both non-denaturing extraction (open symbols) and guanidine-HCl extraction/renaturation (closed symbols) from the pET-15b expression system described in Materials and Methods. Closed squares represent rIL6 (specific activity 10 U/ng) purchased from Life Technologies.

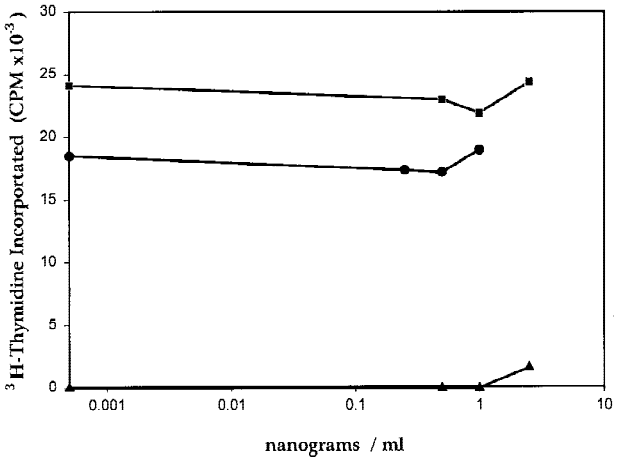

Fig. 4. Effect of rIL6-alt on the proliferative activity of rIL6-full on 7TD1 cells. Cells were incubated 66 hr in the presence of varying concentrations of rIL6-alt in the presence of 50 pg/ml (squares), 25 pg/ml (circles) or 0 pg/ml (triangles) rIL6-full (Life Technologies). ^{3}H-thymidine incorporation (6 hr) was then measured as described in Materials and Methods.

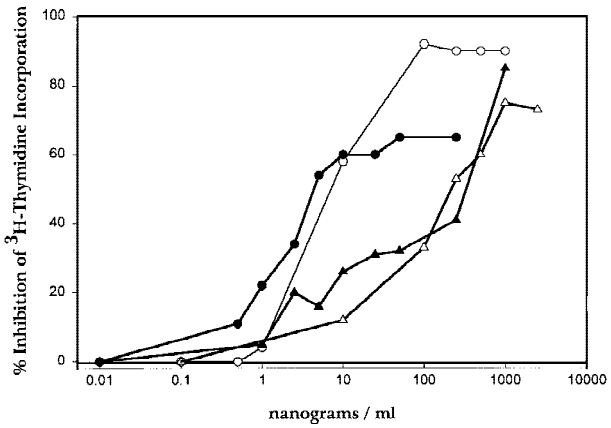

Fig. 5. Antiproliferative activity of rIL6-alt on the M1 murine myeloblast cell line. M1 cells were incubated 66 hr in the presence of recombinant proteins followed by 6 hr culture with ^{3}H-thymidine and assay of incorporation into DNA. rIL6-alt (triangles) and rIL6-full (circles) were purified using both non-denaturing extraction (open symbols) and guanidine-HCl extraction/renaturation (closed symbols) from the pET-15b expression system described in Materials and Methods.

In order to test the hypothesis that IL6-alt might be a natural inhibitor of IL6-full, we examined the effect of mixtures of rIL6-full and rIL6-alt on 7TD1 proliferation (Fig. 4). The rIL6-alt showed no inhibitory effect on 7TD1 proliferation by rIL6-full up to a concentration of 5 ng/ml rIL6-alt (200:1 ratio of rIL6-alt:rIL6-full).

Antiproliferative Activities of rIL6-full and rIL6-alt Toward M1 Murine Myeloblast Cells and HL-60 Human Promyelocytic Leukemia Cells

IL6 is known to inhibit growth and induce differentiation of myeloid cell lines [22]. Therefore, we compared the effects of rIL6-full and rIL6-alt on the growth of murine M1 (myeloblast) and human HL-60 (promyelocytic leukemia) cell lines. Figure 5 depicts a representative experiment with M1 cells and shows that, in contrast to the effect on 7TD1 cells, both recombinant proteins had antiproliferative activity toward M1 cells in the ng/ml range. In 6 separate experiments comparing concentrations required for 50% inhibition of M1 cells, we found that rIL6-full was 10–200 fold more active than rIL6-alt.

In assays using HL-60 promyelocytic leukemia cells (Fig. 6), in contrast to murine M1 myeloblasts, rIL6-alt had appreciable antiproliferative activity in the 0.2–2 μg/ml range whereas rIL6-full had no appreciable activity (less than 5% inhibition) up to 2 μg/ml. Interestingly, in contrast to murine M1 cells and 7TD1 cells, rIL6-alt was *more* active than rIL6-full when tested as an antiproliferative agent against human HL-60 promyelocytic leukemia cells.

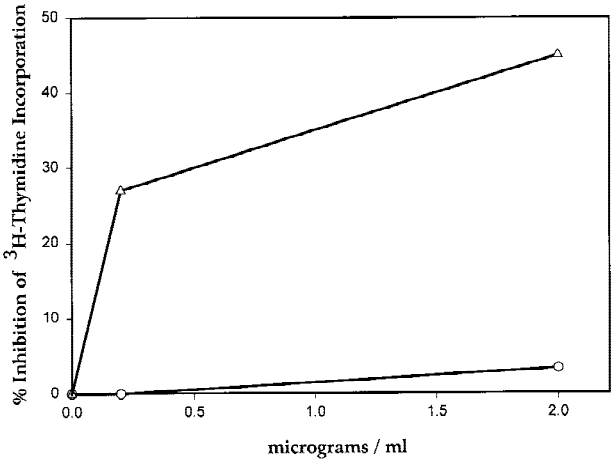

Fig. 6. Effect of rIL6-full and rIL6-alt on the proliferation of HL-60 cells. HL-60 cells were incubated 66 hr in the presence of various amounts of rIL6-full (circles) or rIL6-alt (triangles) followed by assay of ^{3}H-thymidine incorporation (6 hr) as described in Materials and Methods.

Effect of rIL6 and rIL6-alt on Surface Expression of CD11b Antigen in HL-60 Cells

Both rIL6-full and rIL6-alt were evaluated for their ability to induce monocytic differentiation of HL-60 cells as measured by up-regulation of the monocytic differentiation antigen CD11b [23]. Figure 7 shows that rIL6-alt induced expression of CD11b in over 30% of the cells after 72 hr of culture. In contrast, rIL6-full induced less than 7% of the cells to express CD11b antigen. In control experiments, polymyxin-B (10 μg/ml) did not inhibit the effect of rIL6-alt on CD11b expression, suggesting that

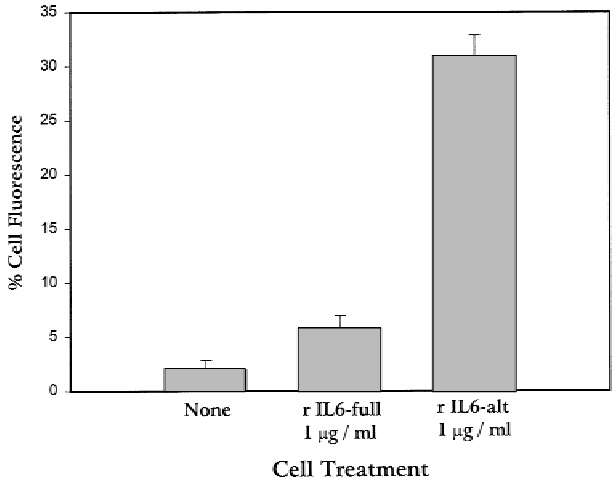

Fig. 7. Effect of rIL6-full and rIL6-alt on the surface expression of CD11b antigen in HL-60 cells. HL-60 cells were cultured for 72 hr in the presence and absence of 1 μg/ml rIL6-full or rIL6-alt, followed by indirect immunofluorescence/ flow cytometry analysis of CD11b expression as described in Materials and Methods. Bars represent mean ± SD (error bars) of three separate experiments.

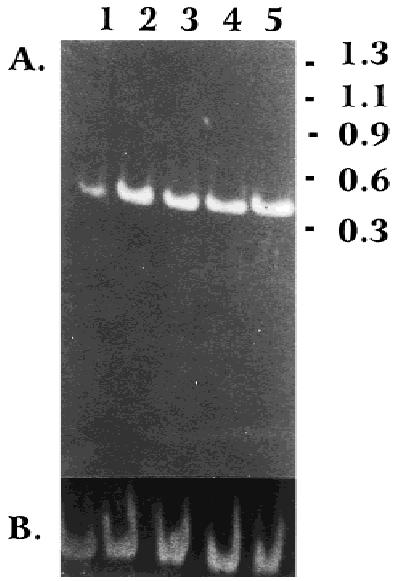

Fig. 8. Effect of rIL6-full and rIL6-alt on the accumulation of lysozyme mRNA in HL-60 cells. HL-60 cells were cultured for 16 hr in the presence and absence of various additions as described in Materials and Methods. RNA was then extracted and equivalent amounts were submitted to RT-PCR analysis by using either lysozyme (panel A) or β-actin (panel B) oligonucleotide primers. The lane assignments are: (1) no addition; (2) 1% DMSO; (3) 50 ng/ml phorbol myristate acetate (PMA); (4) 1 μg/ml rIL6-full; (5) 1 μg/ml rIL6-alt. Molecular weight markers in kbp are indicated along the right margin.

contaminating endotoxin was not responsible for the biologic activity of the rIL6-alt preparations (data not shown). Thus, in contrast to its effect on 7TD1 plasmacytoma proliferation and murine M1 myeloblast differentiation, rIL6-alt appears to have a greater effect on HL-60 monocytic differentiation when compared to rIL6-full.

Up-regulation of Lysozyme mRNA by rIL6-full and rIL6-alt in HL-60 Cells

Lysozyme has been shown to be induced in myeloid cells in response to differentiation agents, including IL6 [24]. We therefore evaluated the effects of both rIL6-full and rIL6-alt on lysozyme mRNA levels in HL-60 cells. As shown in Figure 8, rIL6-full and rIL6-alt upregulated lysozyme mRNA approximately equally after 16 hr culture, as assessed by RT-PCR analysis.

Phagocytic Effects of rIL6-full and rIL6-alt on HL60 Cells

In order to examine effects on functional ability, we evaluated the effect of both rIL6-full and rIL6-alt on phagocytic activity of HL-60 cells. As shown in Figure 9, culturing HL-60 cells in the presence of rIL6-alt at 1 μg/ml induced >35% of cells to phagocytose 1 μ latex beads, whereas rIL6-full induced <10% phagocytic cells. Control cells stimulated with PMA, a strong monocytic differentiation agent, induced slightly less phagocytic activity than rIL6-alt. In data not shown, we confirmed these microscopy results by using flow cytometry of HL-60 cells exposed to fluorescent 1 μ latex beads.

DISCUSSION

IL6 is a multifunctional cytokine that is active toward B cells, T cells, hematopoietic cells, and hepatocytes [1–4,25–27]. This cytokine has been implicated in the pathogenesis of multiple myeloma and other malignancies, in which IL6 has been postulated to serve as an autocrine growth factor [5,28,29]. IL6 is produced by a wide variety of cell types, many of which are also directly stimulated by IL6, suggesting that some mechanism exists that modulates IL6 activity in various cells. Two possible control mechanisms might involve IL6 inhibitory molecules or relative tissue specificity, which might be the result of differential IL6 receptors and/or signal transducing mechanisms. Previously, we reported the identification and initial characterization of an alternatively spliced isoform of human IL6 which lacked the gp130 interactive exon II encoded portion of IL6 (13). The IL6-alt mRNA was originally identified by using RT-PCR and the cDNA subsequently cloned. We also detected in cell lysates a lower molecular mass IL-6 protein (approximately 17 kd), which might represent the putative protein product of the alternatively spliced mRNA (13). We postulated that IL6-alt might represent a natural inhibitor or serve as a form of IL6 with relative tissue specificity. Based on DNA sequence analysis of

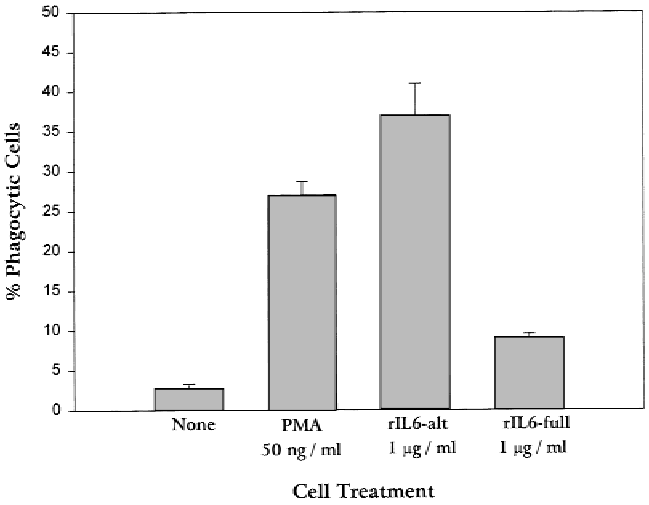

Fig. 9. Effect of rIL6-full and rIL6-alt on phagocytosis by HL-60 cells. HL-60 cells were cultured for 68 hr in the presence and absence of various additions as described in Materials and Methods, followed by 4 hr assay of phagocytic activity by using 1 μ latex beads. Bars represent mean ± SEM of three determinations.

IL6-full [1] and our recently cloned IL6-alt [13], we expressed IL6-alt protein by inserting an ATG start codon before the exon III splice site of IL6-alt (Fig. 1) under the assumption that, like IL6-full, exon I-encoded peptide would be cleaved before secretion of the mature protein [1,14,15]. This would appear to be the most likely protein expressed from the IL6-alt alternatively spliced mRNA, but it is possible that another protein expression mechanism exists in which amino acids encoded by exon I would remain in the IL6-alt protein.

Induced expression of the rIL6-alt polypeptide from pET-15b vector in *E. coli* DE3 cells resulted in large amounts of insoluble recombinant protein in inclusion bodies, similar to what has been reported for rIL6-full expression [14,30,31]. Extraction of this IL6-alt protein with guanidine-HCl followed by dialysis resulted in highly pure rIL6-alt of which <50% bound to DEAE anion exchange resin and possessed bioactivity (data not shown). In contrast and consistent with previous reports from other laboratories, approximately 80% of rIL6-full prepared under similar conditions was recovered with bioactivity after DEAE anion exchange chromatography [15,30,31]. We also prepared rIL6-alt and rIL6-full by detergent extraction without guanidine-HCl and found that although the yields of purified material differed, the activities were similar when assayed against 7TD1 murine plasmacytoma cells (Fig. 3).

In this report, we compare biologic activities of these two recombinant IL6 isoforms. We provide evidence that IL6-alt is relatively tissue specific with higher activity toward myeloid cells and relatively little activity (10^4–10^5-fold less than IL6-full) toward B-cells/plasma cells. The rIL6-alt did not demonstrate competitive inhibitory activity when mixed with rIL6-full in 7TD1 proliferation assays, suggesting that IL6-alt does not serve as a significant natural inhibitor of IL6. The ratio of rIL6-alt:rIL6-full activity was considerably higher in murine myeloid cells as compared to murine plasmacytoma cells. In murine M1 myeloblast cells, rIL6-alt was 10–200 fold less active that rIL6-full, but nevertheless at high doses was capable of inhibiting proliferation >80%. In marked contrast, in assays of antiproliferation and differentiation (phagocytosis, expression of CD11b) of human HL-60 promyelocytic leukemia cells, rIL6-alt was appreciably *more* active when compared to rIL6-full. In agreement with data from other laboratories, in our studies rIL6-full had little or no antiproliferative activity toward HL-60 cells [32], whereas rIL6-alt had modest antiproliferative activity (>30% inhibition at 1 μg/ml). Another marker of monocytic differentiation, stimulation of lysozyme mRNA levels in HL-60 cells [23], was observed to be similar for rIL6-alt and rIL6-full. Phagocytosis, CD11b expression, and lysozyme expression are all considered to be relatively late differentiation markers [24,33]. However, lysozyme mRNA expression is controlled by a complex set of signals. For example, lysozyme expression has been shown to preceed the expression of myeloperoxidase in monocytic differentiation whereas it occurs after myeloperoxidase expression in neutrophilic differentiation [34]. Lysozyme has also been shown to be up-regulated by NF-IL6, a transcriptional factor which up-regulates IL6 expression and can itself be up-regulated by IL6 [35]. Demethylation of the lysozyme gene, which has been correlated with lysozyme expression during differentiation of normal myeloid cells [36], has not been observed during differentiation of myeloid leukemic cell lines such as HL-60 [37].

In conclusion, our studies show that IL6-alt has lost B-cell stimulatory activity associated with IL6-full, while retaining myeloid antiproliferative/differentiation activities. Interestingly, the myeloid antiproliferation/differentiation activity of rIL6-alt toward human HL60 promyelocytic leukemia cells was appreciably more than the activity of rIL6-full. Because IL6-alt lacks the exon II encoded amino acid residues that are known to interact with the signal transducing gp130 molecule on cells [11,12,38,39], we previously hypothesized that IL6-alt might serve as a natural inhibitor of IL6 [13]. However, data presented in this report do not support this hypothesis because rIL6-alt did not inhibit rIL6-full stimulation of 7TD1 plasmacytoma proliferation.

A large body of evidence from many laboratories supports a multi-site model of IL-6 activity with at least one site contributing to receptor binding and at least two additional sites contributing to gp130 interaction for signalling, one of which is not encoded by exon II [1,11,12,38,39]. Other data suggest that the gp130 signal

transduction moiety has separate regions for myeloid growth arrest and differentiation signals (12). This suggests that other functions such as proliferative signals or apoptosis modulation may also be mediated by different regions of the molecule. The data presented here is consistent with such a multi-site model of IL6 because rIL6-alt is diminished by one of the two known gp130 interactive domains.

Other data presented in this report support our alternative hypothesis that IL6-alt is relatively tissue specific and may have an important role in the modulation of hematopoiesis, whereas IL6-full may have a more important role in B cell proliferation and differentiation. It may be that the relative tissue specificity of rIL6-alt vs. rIL6-full is determined in part by species of origin as well as cell type (lymphoid vs. hematopoietic). In a previous report [13] using RT/PCR to examine expression of IL6-alt and IL6-full mRNA in LPS and Con A stimulated peripheral blood lymphocytes, data suggested that IL6-alt is expressed to a greater extent in B cells. This also would be consistent with rIL6-alt having relatively specific tissue distribution and with it having little effect on B cell proliferation since otherwise it would lead to nonspecific expansion of polyclonal B cells. Further studies examining expression of IL6-alt and IL6-full mRNA in different hematopoietic, lymphoid, and other cells are currently in progress. Other work in progress in our laboratory analyzing biologic activities and binding of rIL6-full and rIL6-alt to other human and mouse myelocytic cell lines, peripheral blood monocytes, and bone marrow cells should help to clarify and extend these observations.

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