

IL-6 synthesis by rheumatoid synoviocytes is autonomously upregulated at the transcriptional level

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Background: Involvement of IL-6 in the pathogenesis of rheumatoid arthritis has recently been demonstrated, but the mechanism of its production by rheumatoid synoviocytes is still poorly defined.

Objective: The purpose of this study was to clarify the cellular and molecular mechanisms involved in the spontaneous production of IL-6 by fibroblast-like synoviocytes obtained from patients with rheumatoid arthritis.

Methods: Cloned synoviocytes were established by the limiting dilution method. IL-6 synthesis was evaluated by ELISA and Northern blot analysis. IL-6 gene transcription and transcription factors were analyzed by the transient transfection of luciferase reporter plasmids and the electrophoretic mobility shift assay, respectively.

Results: IL-6 synthesis by cloned rheumatoid synoviocytes was spontaneously upregulated at the transcriptional level.

Enhanced IL-6 production by high-producing clones was independent of cytokines from other cell populations or autocrine production of tumor necrosis factor- α and IL-1. Deletion analysis showed that the IL-6 promoter was regulated by 2 positive elements (–159 to –142 base pair and –77 to –59 base pair). The transcriptional activity of the latter element was upregulated in clones showing high IL-6 production. The binding activity of NF- κ B p50/p65 heterodimer and RBP-J κ was enhanced in these clones.

Conclusion: IL-6 production by rheumatoid synoviocytes is autonomously upregulated at the transcriptional level and spontaneous activation of NF- κ B and RBP-J κ seems to be involved. (*J Allergy Clin Immunol* 1999;103:S437–44.)

Key words: Rheumatoid arthritis, IL-6, synoviocytes, NF- κ B, RBP-J κ

Rheumatoid arthritis is a chronic and systemic inflammatory disease, characterized by the destruction of articular cartilage and bone in its chronic phase.¹ In particular, macrophage-like synoviocytes and fibroblast-like synoviocytes (FLS) of the hyperplastic synovium exhibit an activated phenotype.^{2–4} These cells are a major source of several inflammatory cytokines such as IL-1, TNF- α , and IL-6, which play a crucial role in the pathophysiologic condition of rheumatoid arthritis.^{2,5,6}

IL-6 is a pleiotropic cytokine that exerts a variety of well-established effects, including B-cell and T-cell acti-

Abbreviations used

EMSA: Electrophoretic mobility shift assay

FLS: Fibroblast-like synoviocytes

vation, development of fever, and release of acute-phase reactants.^{7–9} In addition, this cytokine in co-operation with its soluble receptor is involved in the proliferation of synoviocytes from patients with rheumatoid arthritis.¹⁰ Immunologic disorders often associated with rheumatoid arthritis include polyclonal plasmacytosis, production of autoantibodies, increased levels of acute-phase reactants, and an increased platelet count, all of which are related to the biologic actions of IL-6,¹¹ suggesting that it is one of the key cytokines in the development of this disease.

Synoviocytes obtained from patients with rheumatoid arthritis produce biologically active IL-6 in vitro, which enhances B-cell growth, immunoglobulin synthesis, and hepatic production of acute-phase reactants.¹² However, the mechanism underlying the spontaneous production of IL-6 by synoviocytes remains poorly defined. Because previous investigations have used a heterogeneous mixture of adherent synoviocytes containing fibroblast-like cells, dendritic cells, and macrophage-like cells,^{5,12–14} it is still uncertain whether IL-6 is produced autonomously by rheumatoid synoviocytes or is produced in response to proinflammatory cytokines such as TNF- α and IL-1 released from other cell populations. In the present study, we established cloned lines of FLS from patients with rheumatoid arthritis to clarify the cellular and molecular mechanisms of IL-6 production by these cells.

METHODS

Reagents

Recombinant human IL-6 was obtained from Genzyme Corp (Cambridge, Mass). A neutralizing monoclonal antibody for TNF- α (MAB210) was purchased from R&D Systems (Minneapolis, Minn). A neutralizing polyclonal antibody (P-401) for both IL-1 α and IL-1 β was purchased from Endogen (Woburn, Mass). Polyclonal anti-p50 (sc-114X), p52 (sc-848X), p65 (sc-109X), c-Rel (sc-70X), HMG I(Y) (sc-1564) antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). Anti-RBP-J κ antibody was provided by Dr Honjo (Kyoto University, Japan).

Cells

Synovial tissue samples were obtained from 2 patients with rheumatoid arthritis and 1 patient with osteoarthritis who were undergoing total joint replacement. All patients were evaluated by a rheumatologist and were diagnosed according to the criteria of the American College of Rheumatology.^{15,16} Written informed consent

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was obtained from each patient. Each tissue specimen was minced and digested with 4 mg/mL collagenase for 2 hours at 37°C. Cells were plated in RPMI 1640 (Nikken, Kyoto, Japan) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY). When the cells had grown to confluence, they were treated with trypsin/EDTA and split at a 1:4 ratio. For experiments, FLS were used at passages 3 to 10.

Establishment of cloned FLS

Synoviocytes were cloned by limiting dilution as described previously.¹⁷ Briefly, the cells were suspended in RPMI 1640 medium supplemented with 10% FBS at a density of 5 cells/mL. A 100- μ L aliquot of the cell suspension was then inoculated into each well of a 96-well culture plate (0.5 cells/well) and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. No more than 10 wells per plate were positive for growth, which was confirmed to be from a single cell by careful microscopic examination 2 days after cloning. Cloned FLS were expanded up to the third passage under the conditions described earlier.

Measurement of IL-6

Cells at confluence were preincubated for 24 hours in RPMI 1640 supplemented with 0.1% BSA to exclude the effect of FLS. The medium was then exchanged for fresh medium, and the cells were further cultured for 6 hours. The culture supernatants were stored frozen until IL-6 was assayed by a specific sandwich ELISA. Briefly, supernatants or serial dilutions of recombinant IL-6 standards (Genzyme) were incubated overnight at 4°C in 96-microtiter plates (Nunc, Roskilde, Denmark), which had been precoated overnight at 4°C with an anti-human IL-6 monoclonal antibody (2 μ g/mL; R&D Systems, Minneapolis, Minn). After the plates had been washed, a biotinylated anti-human IL-6 polyclonal antibody (2 μ g/mL) (R&D Systems) was added, and incubation was done for 4 hours at room temperature. After subsequent incubation for 2 hours at room temperature with horseradish peroxidase-conjugated avidin (Zymed Laboratories, Inc, San Francisco, Calif), 3,3',5,5'-tetramethylbenzidine (Dojindo Labs, Kumamoto, Japan) was added to the wells, and the absorbance at 450 nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, Calif). The detection limit of the assay was 6.25 pg/mL.

Northern blotting

Cells were grown to confluence in 175-cm² culture flasks and then harvested. Total RNA was prepared from the cells by the method of Chomczynski and Sacchi.¹⁸ Twenty micrograms of total RNA was loaded onto a 1.2% (wt/vol) agarose gel-containing formaldehyde for electrophoresis and then was transferred to Hybond N nylon membranes (Amersham, Little Chalfont, UK) with a VacuGene XL vacuum blotting apparatus (Pharmacia/LKB, Piscataway, NJ). Specific mRNAs were detected by hybridization with appropriate randomly primed ³²P-labeled cDNA probes (BcaBEST Labeling Kit; Takara, Osaka, Japan). The probe for human IL-6 cDNA was a 1.16-kb *EcoR* I/*EcoR* I fragment (ATCC; Rockville, Md)¹⁹ and for human GAPDH cDNA was a 0.8-kb *Pst* I/*Xba* I fragment (ATCC).²⁰ Autoradiograms of the Northern blots were quantified by scanning densitometry.

Plasmid construction

For generation of the pIL6-2BLuc plasmid, a *Sac* I/*Xho* I fragment containing the 1.2-kbp *Bam*HI/*Xho*I 5' upstream sequence of the IL-6 gene was excised from the pGEMhIL-6 GT plasmid (Riken Gene Bank, Tsukuba, Japan)²¹ and inserted into the compatible sites (*Sac*I/*Xho*I) of a pGL2-Basic vector plasmid (Promega Corp, Madison, Wis).

For generation of the pIL6-3BLuc plasmid, a *Sac*I/*Xho*I fragment containing the 1169-bp (–1158 to +11 relative to the transcription initiation site) *Bam*HI/*Xho*I 5' upstream sequence of the IL-6 gene was excised from the pGEMhIL-6 GT plasmid (Riken Gene Bank) and inserted into the compatible site (*Sac*I/*Xho*I) of the luciferase reporter plasmid pGL3-Basic (Promega Corp). A series of deletion mutants of the 5'-flanking region of the IL-6 gene were created as follows: For the construction of pIL6NX-3BLuc, pIL6-3BLuc was digested with *Nhe*I and *Xho*I to generate the *Nhe*I/*Xho*I (–225 to +11) fragment of the IL-6 upstream region, which was then cloned into pGL3-Basic. For the construction of pIL6BX-3BLuc, pIL6AX-3BLuc, pIL6HX-3BLuc, and pIL6SX-3BLuc, pIL6B-3BLuc was digested with *Bfa*I, *Aat*II, *Hae*III, or *Ssp*I, respectively, blunt-ended with Klenow enzyme or T4 polymerase, and released by *Xho*I digestion. The resulting fragments were subcloned into the pBluescript II KS(+) phagemid vector (Stragagene, La Jolla, Calif), which was digested with *Sac*I and *Xho*I. Then the *Sac*I/*Xho*I fragment was inserted into pGL3-Basic.

The reporter constructs (IL6- κ B $\times$ 4, C/EBP β $\times$ 4, and AP-1 $\times$ 4) were generated by cloning 4 copies of the corresponding oligonucleotides as direct repeats into *Kpn*I and *Sac*I sites of the pGL3-tk vector, which carries a truncated thymidine kinase promoter²² connected to the luciferase reporter gene. The DNA sequences of the oligonucleotides used were as follows: IL6- κ B, GATCATGTGGGATTTCCTCAT; C/EBP β , GATCACATTGCACAATCT; and AP-1, GATCAGATTCTAGGAATTCAA. The oligonucleotides were synthesized with *Kpn*I and *Sac*I overhangs.

Transient transfection and luciferase assay

Cells were grown to confluence in 175-cm² flasks containing RPMI 1640 medium supplemented with 10% FBS, harvested, and resuspended in RPMI 1640 without phenol red at a concentration of 2×10^6 cells/mL. A 500- μ L aliquot of the cell suspension, 10 μ g of reporter plasmid, and 1 μ g of pCMV- β -gal were placed into a 4-mm cuvette (Bio-Rad Laboratories); and electroporation was performed (Gene Pulser, 0.29 kV, 960 μ F; Bio-Rad Laboratories). Then the transfected cells were cultured for 18 hours in RPMI 1640 without phenol red in 96-well plates (Culture Plate; Packard Instrument Company, Meriden, Ct). The luminescence was measured with a Top Count (Packard Instrument Company) with a counting time of 0.15 minutes, a single photon counting mode, and a 15-minute period of dark adaptation at 22°C. β -galactosidase activity was measured as described previously.²³ Briefly, cells were washed with PBS(–) and then 100 μ L of 1.5 mmol/L chlorophenol red- β -D-galactopyranoside (Boehringer Mannheim, Mannheim, Germany) in PBS(–) containing 20 mmol/L KCl, 2 mmol/L MgSO₄, 100 mmol/L 2-mercaptoethanol, and 0.5% Nonidet P-40 (Wako, Osaka, Japan) was added; β -galactosidase activity was determined by measuring the absorbance at 570 nm with a microplate reader (Bio-Rad Laboratories).

Preparation of nuclear extracts

Nuclear extracts were prepared by the method reported previously²⁴ with slight modifications. The cells were washed with ice-cold PBS(–), harvested, and resuspended in 400 μ L of hypotonic buffer A (10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenyl methyl sulfonyl fluoride) for 15 minutes on ice. The cells were then lysed in 0.6% nonidet P-40 by vortexing for 10 seconds. Nuclei were separated from the cytosol by centrifugation at 12,000g for 30 seconds, washed with 400 μ L of buffer A containing 0.6% nonidet P-40, resuspended in buffer C (20 mmol/L HEPES [pH 7.9], 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L phenyl methyl sulfonyl fluoride), vigorously vortexed for 15 seconds, and incubat-

TABLE I. Spontaneous IL-6 production by cloned FLS obtained from patients with rheumatoid arthritis

Clone*	IL-6 (pg/mL) [†]
RA14.1	550 ± 46
RA14.2	115 ± 5
RA14.3	807 ± 14
RA14.4	770 ± 20
RA14.5	1100 ± 12
RA15.1	1372 ± 10
RA15.2	353 ± 12
RA16.1	225 ± 9
RA16.2	559 ± 19
RA16.3	1621 ± 70

*Synoviocytes were cloned by the limiting dilution method and then were incubated in 24-well culture plates at a density of 3×10^3 cells/cm² for 6 hours.

[†]The IL-6 concentration in culture supernatant was assayed by a specific ELISA. The IL-6 concentration in culture medium (0.1% BSA/RPMI 1640) alone was below the detection limit. Data are shown as the mean ± SD of quadruplicate cultures.

ed for 5 minutes on ice. This step was repeated 3 times. Then nuclear extracts were obtained by centrifugation at 12,000g for 10 minutes. The protein concentration was essentially measured by the method of Bradford²⁵ with a protein dye reagent (Bio-Rad Laboratories).

Electrophoretic mobility shift assay

The double-stranded oligonucleotide was used for electrophoretic mobility shift assay (EMSA). A probe was derived from the following sequence 5' upstream of the transcriptional start site of human IL-6 gene: IL6-κB, 5'-tcgacATGTGGGATTTTCCCAT-GAc-3' (-77 to -59). DNA binding and the EMSA were performed as described previously.²⁶ The 3' ends of the oligonucleotides were labeled with [α -³²P]dCTP with Klenow DNA polymerase (Megaprime DNA labeling systems, Amersham). Samples of 10 μ L containing 5 μ g of nuclear extract were incubated with 10,000 cpm of oligonucleotides labeled for NF-IL6 and IL6-κB and 1 μ g of poly(dI-dC) in 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, and 5% glycerol. The samples were incubated in the presence or absence of competitor oligonucleotides for 15 minutes at room temperature and run on 4% polyacrylamide gels in 0.5 × TBE at 150 V.

Statistics

Statistical analysis was performed by ANOVA and Scheffé's *F* test on a StatView 4.0 software program (Abacus Concepts, Inc, Berkeley, Calif). *P* < .01 was considered significant.

RESULTS

FLS from patients with rheumatoid arthritis spontaneously produced significantly larger amounts of IL-6 than those from the osteoarthritis patient (rheumatoid arthritis patient 1, 1620 ± 70 pg/mL; [*P* < .001; *n* = 3]; rheumatoid arthritis patient 2, 1975 ± 30 pg/mL [*P* < .001; *n* = 3] vs osteoarthritis patient, 202 ± 52 pg/mL). To clarify cellular requirements for IL-6 production by rheumatoid FLS, we established cloned synoviocytes by the limiting dilution method and measured the amount of IL-6 produced by these clones. Some of the FLS clones (RA14.1, RA14.3, RA14.4, RA14.5, RA15.1, RA16.2,

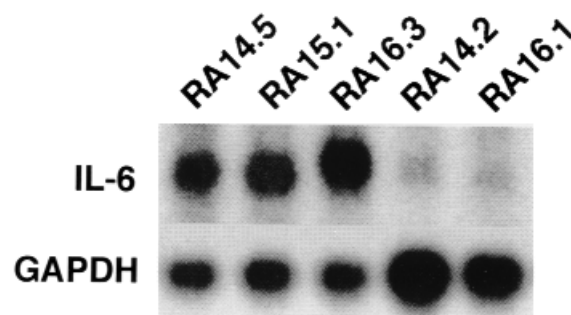


FIG 1. Enhanced IL-6 mRNA expression by cloned rheumatoid FLS. Total cellular RNA was extracted from cloned rheumatoid FLS. Total RNA (20 μ g) was fractionated on 1.2% (wt/vol) agarose gels, transferred to Hybond N nylon membranes, and hybridized to the appropriate cDNA probes.

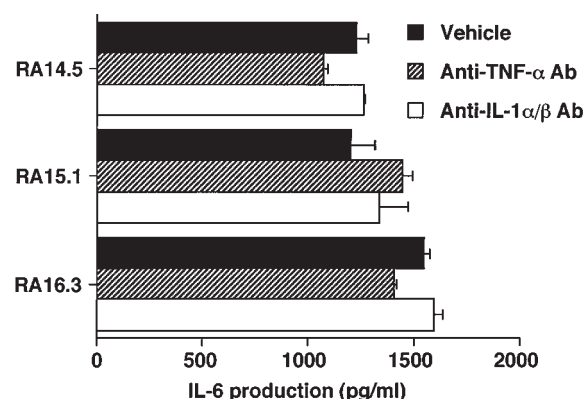


FIG 2. Effects of anti-TNF- α and anti-IL-1 antibodies on the spontaneous production of IL-6 by high IL-6-producing rheumatoid FLS clones. The clones (RA14.5, RA15.1, and RA16.3) were preincubated in 24-well culture plates at a density of 3×10^3 cells/cm² for 18 hours in the presence or absence of neutralizing antibodies (10 μ g/mL anti-TNF- α and anti-IL-1), and then suspended in fresh medium containing the same antibodies for 6 hours. The IL-6 concentration in the culture medium was assayed by a specific ELISA. The IL-6 concentration in the culture medium itself (0.1% BSA/RPMI 1640) was below the detection limit. Data are shown as the mean ± SD of quadruplicate cultures.

and RA16.3) spontaneously showed high levels of IL-6 production (Table I), whereas other clones (RA14.2, RA15.2, and RA 16.1) produced little IL-6. Northern blot analysis indicated that IL-6 mRNA expression in the high IL-6-producing clones (RA14.5, RA15.1, and RA16.3) was significantly greater than in the low IL-6-producing clones (RA14.2 and RA16.1; Fig 1).

Because there was a possibility that spontaneous production of IL-6 could be caused by proinflammatory cytokines (TNF- α , IL-1 α , and IL-1 β) released from the FLS themselves, the effects of TNF- α and IL-1 α/β neutralizing antibodies on IL-6 production were analyzed. Neither anti-human TNF- α nor IL-1 α/β antibody affected IL-6 production (Fig 2), nor did the combination of antibodies (data not shown). The concentrations of the neutralizing antibodies used were prepared according to the manufacturers' instructions, and antibodies at 10

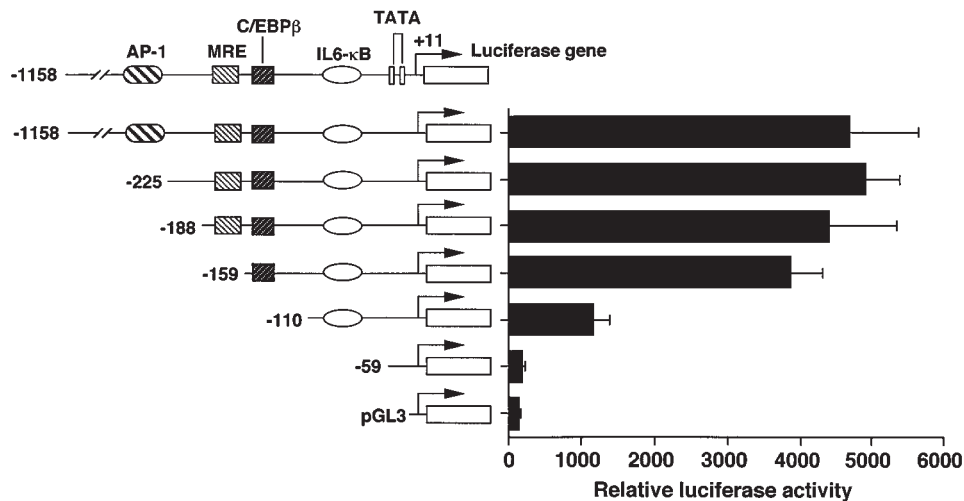


FIG 3. Deletion analysis of the 5'-flanking region of the human IL-6 gene. Putative consensus sequences in the 5'-upstream region of the gene are illustrated in the upper left. Each deleted promoter fragment was ligated into the luciferase reporter plasmid pGL3-Basic. Numbers indicate distances in base pairs from the transcription start site. Luciferase (10 μ g) and β -galactosidase (1 μ g) plasmids were cotransfected into rheumatoid FLS by electroporation. The transfected cells were incubated for 18 hours. Then luciferase activity was assayed and expressed after normalization by the β -galactosidase activity. Data are shown as the mean $\pm$ SD of triplicate cultures.

TABLE II. Transcriptional activity of the human IL-6 gene expressed in the cloned rheumatoid FLS

Clone*	Relative luciferase activity†	
	pGL2-Basic	pIL6-2B
RA14.2	129 $\pm$ 28	353 $\pm$ 73
RA14.5	90 $\pm$ 18	1275 $\pm$ 261‡
RA16.3	44 $\pm$ 17	1305 $\pm$ 82‡

*FLS were transiently transfected with 10 (μ g of pGL2-Basic or pIL6-2B) by electroporation. The synoviocytes were cotransfected with 1 μ g pCMV- β -gal. Then the cells were cultured for 18 hours and harvested.

†Luciferase activity was assayed and expressed after normalization by the β -galactosidase activity. Data are shown as the mean $\pm$ SD of quadruplicate cultures.

‡ $P < .001$ compared with a low IL-6-producing clone (RA14.2).

μ g/mL were sufficient to block IL-6 production by FLS in the presence of 1 ng/mL TNF- α and IL-1 (data not shown).

To determine whether IL-6 synthesis by cloned rheumatoid FLS was upregulated at the transcriptional level, we constructed pIL6-2BLuc, a luciferase reporter plasmid containing the -1158 to +11 human IL-6 promoter region and transfected it into the synoviocytes. High IL-6-producing clones (RA14.5 and RA16.3) exhibited significantly higher luciferase activity compared with the low IL-6-producing clone RA14.2 (Table II). A basic luciferase reporter plasmid, pGL2, which contained no promoter/enhancer gene, was not transcribed by any of the FLS clones. These results clearly indicate that IL-6 synthesis by cloned rheumatoid FLS was enhanced at the level of gene transcription.

As illustrated in Fig 3, approximately 1200 bp of the

5'-flanking region of the human IL-6 gene contains various putative responsive elements: AP-1 (-283 to -277), MRE (-168 to -153), C/EBP β (-155 to -148), IL6- κ B (-73 to -60), and the TATA box (-53 to -47 and -30 to -23).^{11,21,26,27} Functional analysis of the 5'-flanking region was performed with the -1158 to +11 fragment and a series of 5'-deletion mutants of the IL-6 promoter linked to the luciferase reporter gene (Fig 3). Each plasmid was used for transient transfection of rheumatoid FLS by electroporation, and the luciferase activity of the cell lysates was measured 18 hours later. Luciferase activity decreased significantly when two regions (-159 to -142 bp and -77 to -59 bp) were deleted. A C/EBP β homologous element (-155 to -148 bp), an NF κ B homologous element (-73 to -64 bp), and an RBP-J κ homologous element (-67 to -60 bp) were present in these two gene segments. The 5'-CATGGGAA-3' (-60 to -67) sequence was homologous to the RBP-J κ consensus binding element 5'-A(G/C)CGTGGGAA-3'.²⁸ To evaluate which elements were possibly responsible for the constitutive transcriptional activity of the human IL-6 gene expressed in the rheumatoid FLS, we made luciferase reporter gene constructs containing 4 copies of the IL6- κ B, C/EBP β , or AP-1 binding element connected to the thymidine kinase promoter gene (Fig 4, A) and transfected them into both low IL-6-producing and high IL-6-producing clones. Luciferase activity was measured and expressed as the ratio (as a percentage) of activity in high-producing clones to that in low-producing clones. A representative result is shown in Fig 4, B. The luciferase activity of the reporter construct bearing both NF- κ B and RBP-J κ binding elements (IL6- κ B $\times$ 4) was significantly enhanced in high-producing clones

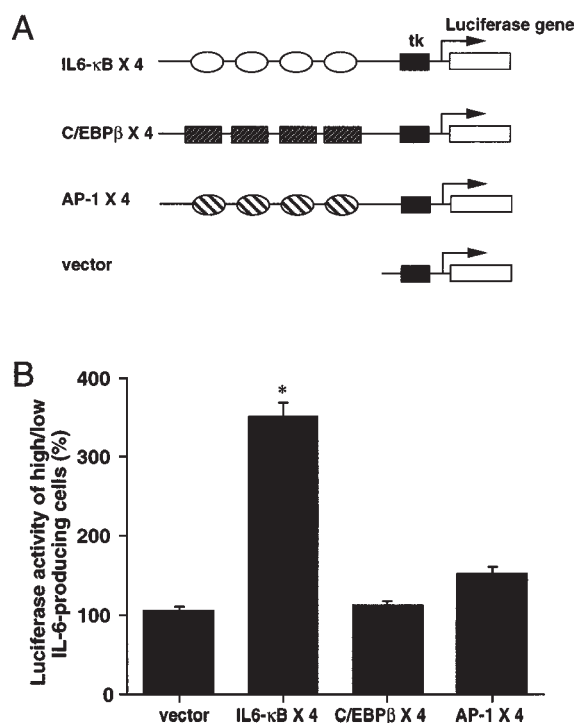


FIG 4. Enhanced transcriptional activity of IL6-κB in high IL-6-producing clones. **A**, Reporter constructs are illustrated. Four copies of the individual oligonucleotides were fused as direct repeats to the thymidine kinase (*tk*) promoter vector pGL3-*tk*, which drives the expression of the luciferase reporter gene. IL6-κB, C/EBPβ, and AP-1 refer to composite elements of the human IL-6 promoter. **B**, Transient transfection of the reporter constructs shown in **A**. High and low IL-6-producing FLS clones were transfected with luciferase (10 μg) and β-galactosidase (1 μg) plasmids by electroporation. The transfected cells were cultured for 6 hours. Then luciferase activity was assayed and expressed as the ratio (as a percentage) of activity in high IL-6-producing clones versus that in low IL-6-producing clones after normalization by the β-galactosidase activity. Data are shown as the mean ± SD of quadruplicate cultures.

compared with that in low-producing clones. In contrast, transcription of the C/EBPβ × 4 and AP-1 × 4 reporter constructs was not significantly different between high- and low-producing clones.

Finally, to determine the transcription factors binding to the IL6-κB element, we analyzed nuclear extracts of high IL-6-producing clones by EMSA. A representative result is shown in Fig 5. At least 2 retarded bands binding to the IL6-κB site were clearly detected (lanes 1 and 7). The binding complexes were specific for each transcriptional element because they were diminished by addition of an excess of unlabeled oligonucleotides (data not shown). We also performed supershift experiments using antibodies for p50, p52, p65, c-Rel, and RBP-Jκ to identify individual proteins in EMSA (lanes 2 to 6 and 8). The slower migrating complex consisted of p50 and p65 NF-κB subunits (lanes 2, 4, and 6) because supershifts were observed with anti-p50 and anti-p65, but not with anti-p52 or anti-c-Rel antibodies. The faster migrating binding complex contained the RBP-Jκ molecule

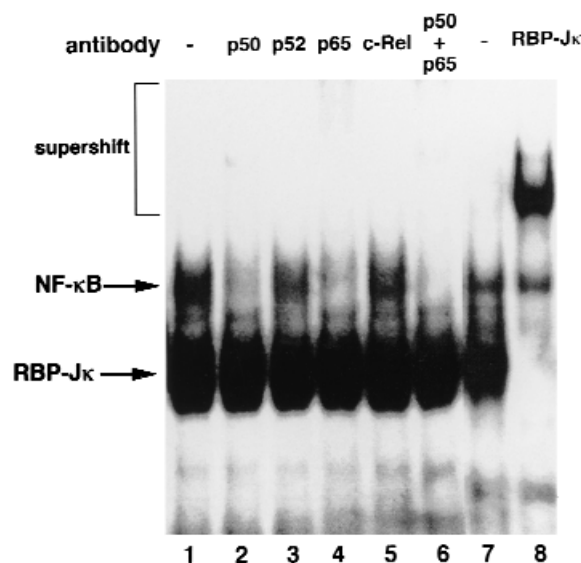


FIG 5. EMSA of binding to the IL6-κB site in nuclear extracts obtained from high IL-6-producing FLS clones. Five micrograms of nuclear extract was incubated with a given ³²P-labeled oligonucleotide probe IL6-κB in the absence or presence of anti-p50, p52, p65, c-Rel, and RBP-Jκ antibodies, and then the DNA-protein binding complexes were visualized on nondenaturing polyacrylamide gel. The arrows indicate the positions of the specific complexes.

because supershifts were observed with the anti-RBP-Jκ antibody.

DISCUSSION

The present study clearly demonstrated that IL-6 production by rheumatoid FLS is autonomously upregulated, because cloned FLS produced large amounts of IL-6 despite the absence of other cell populations. We also demonstrated that IL-6 production was upregulated at the level of gene transcription through spontaneous upregulation of two IL-6 promoter elements at positions -159 to -142 bp and -77 to -59 bp. EMSA and reporter gene analysis indicated that transcriptional factors NF-κB and RBP-Jκ, which bind to the latter positive element, were involved in dysregulation of the IL-6 gene in rheumatoid FLS.

It was recently reported that in vivo blockade of IL-6 signals by the administration of an anti-IL-6 monoclonal antibody or anti-IL-6 receptor antibody was clinically effective,^{29,30} strongly implying that correction of dysregulated IL-6 production may be a rational approach for the treatment of rheumatoid arthritis, even though the pathogenesis of this disease remains obscure. Accordingly, IL-6 production by FLS from rheumatoid joints was investigated in this study.

We first demonstrated that rheumatoid synoviocytes spontaneously produced high levels of IL-6 compared with osteoarthritis FLS. This finding is consistent with the report that rheumatoid synoviocytes spontaneously

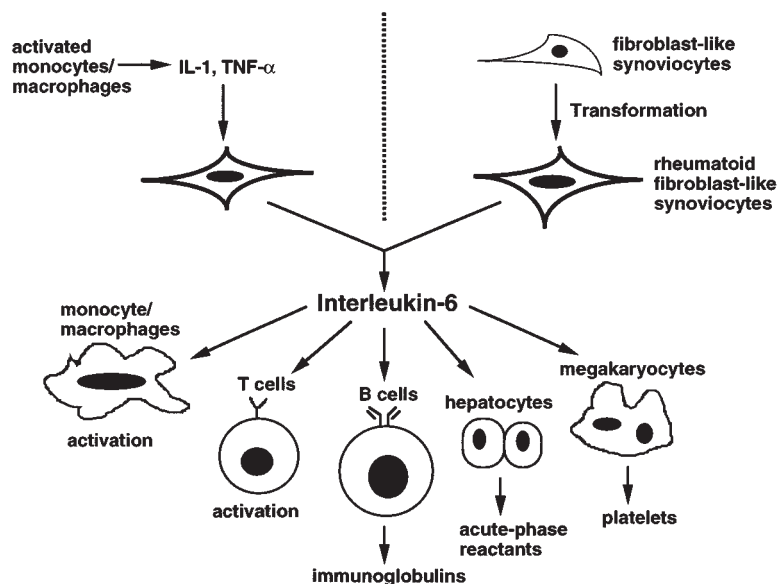


FIG 6. Two possible mechanisms of enhanced IL-6 production by rheumatoid FLS. According to the traditional mechanism, rheumatoid FLS passively produce IL-6 in response to inflammatory cytokines such as TNF- α and IL-1 secreted by macrophages and T cells. Our alternative hypothesis is that rheumatoid FLS are irreversibly altered to autonomously produce high levels of IL-6 even in the absence of known IL-6 inducers.

produce IL-6.¹² However, the possibility remained that IL-6 was produced in response to inflammatory cytokines such as TNF- α and IL-1 possibly secreted from other synoviocyte populations, because these cytokines are potent inducers of IL-6 synthesis *in vitro*.³¹⁻³³ Therefore we established cloned synoviocytes and analyzed the cellular and molecular mechanisms of IL-6 overproduction in the absence of other cell populations.

We found that cloned FLS still showed high IL-6 production. Accordingly, IL-6 production by rheumatoid FLS was autonomously upregulated, because the influence of other inflammatory cell populations, such as lymphocytes, monocytes, and other synoviocytes usually present in the rheumatoid arthritis synovium, was excluded in the present study. The production of IL-6 was not under the autocrine regulation of TNF- α or IL-1, because neutralizing antibodies for these cytokines did not affect spontaneous IL-6 production. This was also confirmed by the fact that neither TNF- α nor IL-1 was detected in the culture medium of high IL-6-producing clones (data not shown).

Reporter gene analysis demonstrated that IL-6 synthesis was upregulated at the transcriptional level because a luciferase reporter plasmid containing the -1158 to +11 human IL-6 promoter region was highly transcribed when transfected into high IL-6-producing FLS clones. This result was supported by the fact that IL-6 mRNA expression by the high-producing clones was significantly enhanced compared with that of the low-producing clones. These findings indicate that spontaneous production of IL-6 by rheumatoid FLS results from the autonomous upregulation of IL-6 gene transcription. Therefore we performed deletion analysis to determine

the *cis*-acting transcriptional elements contributing to enhanced IL-6 gene transcription by rheumatoid synoviocytes.

Transcriptional regulation of the IL-6 promoter involves the interaction of transcription factors such as the NF- κ B/rel family, C/EBP of the bZIP family, cAMP response element-binding protein, and AP-1.¹¹ Because different sets of transcription factors may regulate the IL-6 gene in a cell type-specific manner, it is still uncertain which element is functional in rheumatoid FLS. Therefore we first attempted deletion analysis of the IL-6 promoter and found that the C/EBP β and the IL6- κ B elements were critical for constitutive transcriptional activity of this promoter. Reporter gene analysis clearly demonstrated that transcriptional activity of IL6- κ B was enhanced in the high IL-6-producing clones, whereas the transcriptional activities of C/EBP β and AP-1 were not. Supershift experiments then showed that the IL6- κ B binding complex in the high IL-6-producing clones comprised p50 and p65 NF- κ B subunits. Only very faint expression of the p50 subunit and no expression of the p65 subunit were observed in low IL-6-producing clones (data not shown). Previous studies have established that the p65 subunit of NF- κ B is responsible for transactivation and have shown the existence of a potent transactivation domain in its C-terminal portion.³⁴⁻³⁶ It is thus reasonable that constitutive expression of p65 subunit of NF- κ B would lead to enhanced IL-6 transcriptional activity in rheumatoid synoviocytes. Our finding is consistent with the report that p65 NF- κ B was identified in synovial biopsy specimens obtained from patients with rheumatoid arthritis.^{37,38} In addition, another transcription factor, RBP-J κ , might be involved in the increased IL-6 gene transcription of rheumatoid FLS, because

RBP-J κ -binding activity was also observed in the high IL-6-producing clones. However, further studies are required to define the role of RBP-J κ in IL-6 gene transcription.

In the present study, we clearly demonstrated a high level of autonomous IL-6 production by some rheumatoid FLS. IL-6 increases platelet count and induces the production of acute-phase reactants by hepatocytes.¹³ It also activates T cells and facilitates the differentiation of B cells and may contribute to the production of antibodies (and hence immune complexes) that are proinflammatory.¹¹ Moreover, IL-6 is involved in Fc γ RI expression on monocytes through the induction of STAT family factors.³⁹ Accordingly the overproduction of IL-6 seems to be associated with the immunologic abnormalities of rheumatoid arthritis, and our findings support the following model regarding the regulation of IL-6 production in the rheumatoid arthritis synovium (Fig 6). The traditional concept is that rheumatoid FLS respond passively to inflammatory cytokines such as TNF- α and IL-1 secreted by activated macrophages and T cells. However, we suggest that rheumatoid FLS are irreversibly altered to produce large amounts of IL-6 in the absence of known IL-6 inducers. The cause of such an abnormal transformation of synoviocytes in the rheumatoid arthritis is unknown, but it might be exogenous infectious agents including retroviruses, endogenous substances including collagen and immunoglobulins, and/or endocrine imbalance. The present findings seem to extend our knowledge on the pathophysiologic features of the rheumatoid synovium by demonstrating that rheumatoid FLS show autonomous activation in the absence of an inflammatory environment, although these cells can still respond to proinflammatory cytokines. A greater understanding of the cellular and molecular mechanisms involved in the spontaneous activation of NF- κ B and RBP-J κ may be useful in guiding the design of more effective therapy for rheumatoid arthritis.

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