Interleukin (IL) 4 Differentially Regulates Monocyte IL-1 Family Gene Expression and Synthesis In Vitro and In Vivo

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Summary

Interleukin (IL) 4 is a multifunctional T cell–derived cytokine that inhibits cytokine production and certain effector functions in human monocytes, while enhancing others. We show that IL-4 may contribute to the downregulation and resolution of an inflammatory response by selectively promoting expression of the IL-1 receptor antagonist (IL-1ra) that blocks the action of IL-1. IL-1ra specifically binds to the IL-1 receptor without initiating signal transduction. Peripheral blood monocytes obtained from cancer patients, before and immediately after a regimen of IL-4 immunotherapy, were examined for IL-1ra gene expression. After IL-4 therapy, monocytes from the patients showed a marked increase in IL-1ra mRNA. This selective induction of IL-1ra mRNA in circulating monocytes was reflected by significantly enhanced serum levels of IL-1ra ($p < 0.01$) during IL-4 therapy, which declined after IL-4 treatment. In vitro analysis of IL-4 regulation of monocytes from normal individuals revealed a dose-dependent induction of IL-1ra mRNA within 2–4 h after stimulation without a concomitant effect on the expression of IL-1 mRNA. Increased IL-1ra mRNA was not due to RNA stabilization, but occurred at the level of transcription. In the presence of LPS, IL-4 not only augmented IL-1ra levels, but markedly inhibited LPS-induced IL-1 mRNA expression. The selective upregulation of IL-1ra by resting or activated monocytes, coupled with inhibition of IL-1 production by activated monocytes, as we demonstrate both in vitro and in vivo, suggests that IL-4 may prove clinically useful as a systemic antiinflammatory agent.

The balance between immune responses to infectious agents and antigens and the development of immune-mediated host injury depends, to a large extent, on the elicitation and actions of soluble immunoregulatory cytokines. Cytokines such as IL-2 and IFN-γ, which promote the development of cell-mediated immunity, are derived from Th1 helper T lymphocytes, whereas Th2 cells promote primarily B cell development and function through the production of IL-4, -5, -6, and -10 (for review, see reference 1). Although originally characterized in mice, Th1 and Th2 subsets have recently been identified in humans through the isolation of $CD4^+$ clones from patients with different pathologic profiles (2, 3). Emerging evidence suggests that in addition to regulating the humoral immune response (4, 5), cytokines secreted by Th2 cells may also function to suppress cell-mediated immune pathways normally potentiated by Th1-derived cytokines. For example, IL-4 has been shown to inhibit the production of monokines (6–8), PGE_2, and metalloproteinases induced by IFN-γ and other stimuli (6, 7, 9–11), and by inhibiting the expression of FcγR (12, 13), IL-4 may limit phagocytosis and the release of reactive oxygen intermediates.

These in vitro observations of an antiinflammatory role for IL-4 have recently been extended in studies in vivo. Patients participating in a clinical trial using IL-4 as an anticancer agent displayed altered monocyte functions in which post-therapy monocytes exhibited a significantly diminished ability to secrete PGE_2 and superoxide anion (7). After isolation of the monocytes from the patients receiving IL-4 therapy, the cells recovered their ability to generate IL-1 when challenged in vitro with LPS (7). However, based on recent evidence that IL-4 induces the IL-1 receptor antagonist (IL-1ra)[1] in a monocytic cell line (14), and augments IL-1ra production in LPS-stimulated blood monocytes (15, 16), we subsequently evaluated patient monocytes for IL-1ra gene expression before and after IL-4 therapy. IL-1ra, a 22–25-kD glycosylated protein (17, 18), binds to the human type I and type II IL-1 receptors with similar affinity as IL-1β and IL-1α, but does

[1] *Abbreviations used in this paper:* Cx, cycloheximide; IL-1ra, IL-1 receptor antagonist.

not initiate internalization of the receptor–ligand complex or transduce an activation signal (19). After systemic IL-4 therapy, we found that monocytes from the patients expressed increased mRNA for IL-1ra that was reflected by a 10-fold increase in serum IL-1ra levels. Characterization of the mechanism by which IL-4 regulates IL-1ra mRNA production and peptide secretion revealed rapid and transient induction that was dependent on increased transcription. These data, together with the ability of IL-4 to suppress inducible IL-1 synthesis, implicate IL-4 as a unique systemic antiinflammatory agent.

Materials and Methods

Monocyte Isolation and Culture. PBMC were obtained by leukapheresis (National Institutes of Health Department of Transfusion Medicine) with subsequent centrifugation of the cells through Ficoll-Hypaque (7). Monocyte populations were then purified (90–95% $CD14^+$) by counterflow centrifugal elutriation (20). Monocytes were resuspended in DMEM (Cellgro/Mediatech, Washington, DC) supplemented with 50 μg/ml gentamicin sulfate (Whittaker Bioproducts, Walkersville, MD) and 2 mM L-glutamine (Gibco, Grand Island, NY) and cultured in suspension in 17 × 100-mm polypropylene tubes (Falcon/Becton Dickinson Labware, Lincoln Park, NJ) or as adherent monolayers in 24-well plates (Costar Corp., Cambridge, MA) (10^6/ml) in the absence or presence of recombinant human IL-4 (Genzyme Corp., Cambridge, MA and Immunex Corp., Seattle, WA) which by limulus assay contained <0.06 EU/ml (1 EU = 0.1 ng/ml endotoxin, the limit of detection of the assay; courtesy of Dr. D. Hochstein, Federal Drug Administration, Bethesda, MD). Cultures were then stimulated or not with LPS (LPS, *Escherichia coli* 055.B5, Difco Laboratories, Inc., Detroit, MI) for the indicated time periods.

Patient Populations. Patients with renal cell carcinoma or with malignant melanoma were given rIL-4 (generously provided by Dr. S. Gillis, Immunex Corp., and Dr. E. Bradley, Sterling Pharmaceutical, Malvern, PA; sp act 1.5 × 10^7 U/mg) at doses ranging from 10 to 20 μg/kg body weight three times daily for 3–5 d (7). One day before initiation, during, and at indicated intervals after IL-4 immunotherapy, patients were leukapheresed and the monocytes were purified by elutriation. Monocytes were processed immediately or cryopreserved after isolation enabling pre- and posttherapy cells to be thawed and analyzed simultaneously (7). All patients were treated according to National Cancer Institute Institutional Review Board approved protocols and signed informed consent before treatment.

Northern Blot Analysis. Monocytes were cultured in suspension (10^7/ml in 17 × 100-mm tubes) with or without IL-4, washed with PBS, and total cellular RNA was isolated by using a one-step acid guanidinium-thiocyanate-phenol-chloroform procedure (21). RNA samples (5 μg) were electrophoresed in 1% formaldehyde–agarose gels, blotted onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) and prehybridized at 42°C for at least 4 h before hybridization at 42°C with ^{32}P-labeled human IL-1β (22), IL-1ra (18), (generously provided by Dr. D. Carter, UpJohn Co., Kalamazoo, MI), He7, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (23) cDNA probes. The blots were washed twice in 2× SSC, 0.1% SDS for 15 min at 22°C, and once in 0.1× SSC, 0.1% SDS for 30 min at 65°C. After washing, the filters were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 1–24 h. Scanned images were obtained using a PhosphorImager and Image Quant software (Molecular Dynamics), transferred and reproduced using Macintosh Adobe Photoshop, Image and Aldus Pagemaker (Apple Computer, Inc., Cupertino, CA) software and printed on a color printer (Phaser CP; Tektronix, Inc., Beaverton, OR).

Nuclear Run-off Assays. Monocytes (5 × 10^7) were cultured in the presence or absence of IL-4 (1,000 U/ml) and nuclei were isolated by lysis of the cells in 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 3 μM DTT, 0.3 M sucrose and 0.5% Triton X-100 and disruption with a Dounce homogenizer (size B; Wheaton Scientific, Millville, NJ). The disrupted cells were then centrifuged at 3000 *g* for 10 min at 4°C through a cushion of 30% sucrose and 0.5% Triton X-100 to pellet the nuclei. For the runoff assay, 100 μl of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl and 5 μM DTT) with 100 mM ATP, CTP, and GTP, and 100 μCi α-[^{32}P] UTP (>760 Ci/mmole, Amersham Corp., Arlington Heights, IL) was added to each sample of nuclei. After incubation with continuous shaking at 30°C for 30 min, nuclear transcripts were purified (21) and hybridized at 62°C for 36 h to nitrocellulose filters on which 5 μg of linearized, denatured plasmid DNA had been immobilized. The filters were then washed twice for 30 min in 2× SSC, 0.1% SDS at 62°C, treated with RNase A (10 μg/ml) in 2× SSC for 30 min at 37°C, and washed with 2× SSC, 0.1% SDS for 30 min at 37°C. The filters were exposed to a phosphor screen for 3–16 h and phosphorimages were scanned.

IL-1ra ELISA. IL-1ra peptide was detected using an ELISA kit purchased from R&D Systems, Inc. (Minneapolis, MN).

Results

IL-1ra Is Increased in Cancer Patients Receiving rIL-4 Immunotherapy. As part of a treatment protocol, recombinant human IL-4 was administered intravenously to cancer patients three times daily for 3–5 d. PBMC were obtained 1 d before IL-4 and immediately after cessation of IL-4 administration. After elutriation of the PBMC, the monocytes (>90% $CD14^+$) were cultured and RNA was isolated for Northern analysis with the IL-1 and IL-1ra cDNA probes. Although low levels of IL-1ra mRNA were sometimes detectable in monocytes from the cancer patients before therapy as represented in Fig. 1, a substantial increase in IL-1ra gene expression was observed in most patients after IL-4 therapy. In contrast, when the same mRNA was probed for IL-1β, little or no IL-1β mRNA was detected before or after IL-4 therapy (Fig. 1). Thus, exposure of circulating monocytes to IL-4 in vivo results in a selective augmentation of gene expression for IL-1ra without a concomitant increase in IL-1β mRNA.

To determine if there was a corresponding increase in IL-1ra protein in these patients, serum samples were collected before, during, and 3–12 wk after IL-4 therapy. These serial samples provided internal controls for each patient. Using an IL-1ra–specific ELISA, a striking increase in IL-1ra protein was measured in the serum of the majority of patients during the course of IL-4 therapy (Fig. 2). Whereas pretherapy levels for IL-1ra were only 1.3 ± 0.23 ng/ml, the levels increased 10-fold during therapy (13.54 ± 3.29; $n = 7$; $p < 0.01$). The increase in serum IL-1ra was transient, since serum collected from the same patients 3–12 wk after therapy contained IL-1ra at or near pretherapy levels (1.9 ± 0.67 ng/ml). In those patients whose serum and monocyte mRNA were both available for evaluation, a correlation existed between these two parameters. Patients with increased IL-1ra mRNA also expressed high serum IL-1ra levels (patients 1, 2, 3, and 5 in

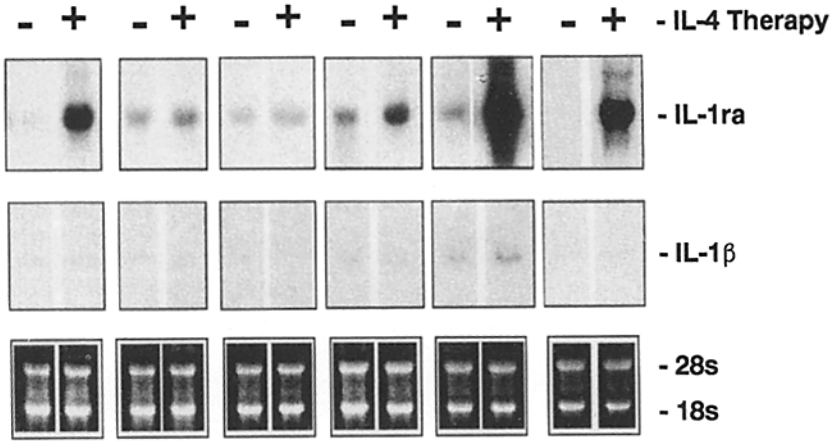

Figure 1. Monocytes from cancer patients given IL-4 express increased IL-1ra mRNA. Melanoma and renal cell carcinoma patients were given rIL-4 (10–20 μg/kg) intravenously for 5 d. Monocytes (2×10^7) were isolated from six of these patients before (−) and within 24 h after (+) IL-4 treatment, cultured, and total cellular RNA isolated for Northern analysis with the IL-1ra and IL-1 cDNA probes. RNA was monitored by ethidium bromide staining of the 18s and 28s ribosomal RNA bands to determine equivalent loading of RNA in each lane.

Fig. 1 had 27.52, 7.03, 5.65, and 20.46 ng/ml serum IL-1ra, respectively).

IL-4 Directly Induces IL-1ra Gene Expression and Peptide Secretion. Since monocytes obtained from patients on IL-4 therapy expressed substantially augmented gene expression for IL-1ra, we examined IL-4 regulation of monocyte IL-1ra in vitro. Elutriated monocytes from normal individuals were incubated with varying concentrations of IL-4 for 4 h at 37°C. As can be seen in Fig. 3 *A*, IL-4 induced detectable gene expression for IL-1ra at concentrations as low as 0.1 ng/ml (Genzyme Corp.) or 10 U/ml (Immunex Corp.), which increased with higher concentrations of IL-4. When the IL-4 was preincubated with a rabbit polyclonal neutralizing antibody to IL-4 (Genzyme Corp.), the IL-4–induced increase in monocyte expression of IL-1ra transcripts was significantly inhibited (data not shown).

To establish whether the IL-4–induced IL-1ra gene expression was accompanied by IL-1ra secretion, monocytes were cultured with or without increasing concentrations of IL-4 for 12–24 h. The supernatants were harvested and IL-1ra was assayed in the supernatants by ELISA. IL-4 caused a dose-dependent increase in the amount of IL-1ra detected in the culture supernatants which plateaued at 12 h (Fig. 3*B*).

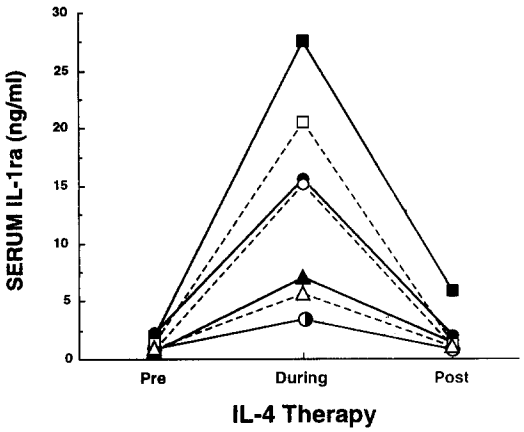

Figure 2. Serum levels of IL-1ra increase in patients during IL-4 therapy. Sera were obtained from patients 1–3 d before administration of IL-4 (*pre*), during IL-4 treatment (days 3–5), and 3–12 wk after treatment (*post*). The sera were diluted (1:5) with buffer and assayed for IL-1ra protein by ELISA.

Differential Regulation of IL-1ra and IL-1 mRNA. Although monocytes exposed to IL-4 in vivo or cultured with IL-4 in vitro demonstrate increased expression of IL-1ra mRNA, no IL-1β mRNA could be detected (Fig. 1 and Fig. 4) indicating independent and selective regulation of IL-1ra. In the presence of LPS, which induces both IL-1 and IL-1ra (24, Fig. 4), IL-4 differentially regulates these two members of the IL-1 family. While augmenting IL-1ra mRNA expression, IL-4 dramatically suppresses LPS-induced IL-1β mRNA (Fig. 4). The net effect of these actions of IL-4 is to neutralize IL-1, both by inhibiting IL-1 production and promoting synthesis and secretion of its antagonist.

Kinetics of IL-1ra Expression. Based on the ability of IL-4 to independently induce only the IL-1ra member of the IL-1 family, we further explored the mechanism of IL-1ra regulation. The kinetics associated with the IL-4–induced expression of IL-1ra mRNA were determined by culturing elutriated monocytes in the absence or presence of IL-4 (1 ng/ml) for 30 min up to 24 h. Total cellular RNA was isolated and by Northern analysis, monocytes exposed to IL-4 exhibited a time-dependent increase in the level of IL-1ra gene expression with peak levels evident within 2–4 h (Fig. 5). Increased expression was transient as mRNA levels subsided and returned to near baseline by 24 h. Densitometric analysis of the IL-1ra signals reflected this increase in transcripts which plateaued within 2–4 h and subsequently declined (Fig. 5).

Regulation of IL-1ra mRNA by IL-4. To investigate potential molecular mechanisms whereby IL-4 induces increased IL-1ra transcripts, the influence of IL-4 on stability of transcribed mRNA was examined. After a 4-h incubation with IL-4 (1 ng/ml), actinomycin D (5 μg/ml), an inhibitor of mRNA transcription, was added to the cultures and at increasing intervals, the cultures were processed for Northern analysis. As shown in Fig. 6 *A*, the IL-4–induced increase in IL-1ra mRNA (fivefold) demonstrated a time-dependent decrease after the addition of actinomycin D, reflecting RNA degradation. Although control (non-IL-4 treated) monocytes

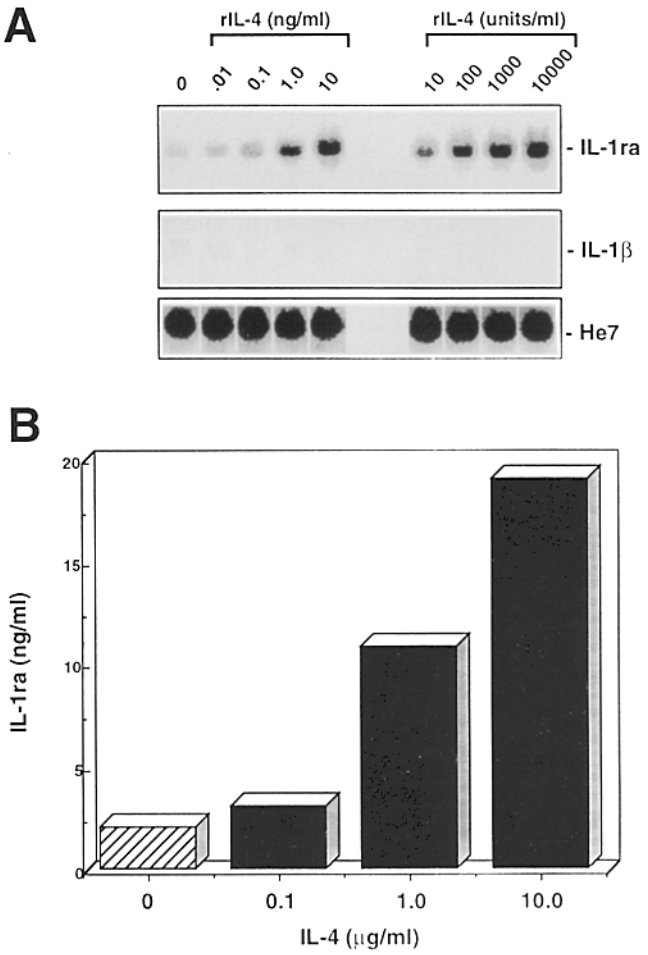

Figure 3. IL-4 induces monocyte expression of IL-1ra mRNA and protein. (*A*) Monocytes (2×10^7) were cultured with IL-4 (0.01–10 ng/ml [Genzyme Corp.] or 10–10,000 U/ml [Immunex Corp.]) for 4 h and RNA was extracted and processed for Northern analysis with the IL-1ra and IL-1β cDNA probes. Equivalent loading of lanes was monitored by constitutive expression of the He7 housekeeping gene. (*B*) Supernatants from monocytes (10^6/ml) cultured with IL-4 (0–10 ng/ml [Genzyme Corp.]) in 24-well culture plates at 37°C for 12 h were harvested, filtered, and assayed for IL-1ra peptide by ELISA.

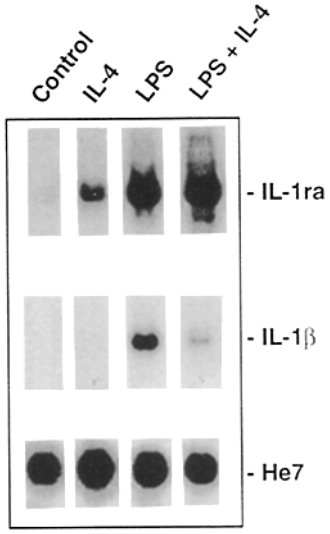

Figure 4. Differential regulation of IL-1 and IL-1ra by IL-4. Monocytes were cultured in suspension with IL-4 (1,000 U/ml) only, LPS (1 μg/ml), or LPS with IL-4. RNA was harvested from the cells after 4 h and processed for Northern analysis with the IL-1ra and IL-1β cDNA probes. Equivalent loading of the lanes was monitored by the constitutive expression of the He7 housekeeping gene.

generally do not constitutively express detectable steady-state levels of IL-1ra mRNA, occasional donor cells were positive for IL-1ra transcripts. To compare the message stability in the absence and presence of IL-4, the data represented in Fig. 6 are from a donor with low, but detectable levels of uninduced IL-1ra mRNA. No differences were observed in the half-life of the mRNA irrespective of the addition of IL-4. When the mRNA bands were analyzed densitometrically and the resulting values plotted vs time, the approximate half-life of IL-1ra calculated from the decay curves was 2.5–3 h (Fig. 6 *A*). These data suggest that IL-4 does not increase the amount of IL-1ra mRNA only through a stabilization of the mRNA molecule.

IL-1ra gene expression was further examined in the presence of cycloheximide (Cx) which, by inhibiting peptide bond formation, prevents protein synthesis. Although the addition of Cx (0.5 or 1.0 μg/ml) did not augment IL-1ra mRNA in the absence of IL-4, the inclusion of Cx with IL-4 increased expression of IL-1ra mRNA two- to threefold above the levels observed for IL-4 alone (Fig 6 *B*). According to these data, a short-lived or labile protein may be involved in the regulation of IL-1ra expression.

To document a transcriptional regulation of IL-1ra by IL-4, nuclear runoff experiments were performed in which nuclei from monocytes cultured with either medium alone or IL-4 were allowed to synthesize mRNA for a defined period of time in vitro. The resulting transcripts were then hybridized to cDNA probes (pGEM vector control, IL-1ra, and GAPDH)

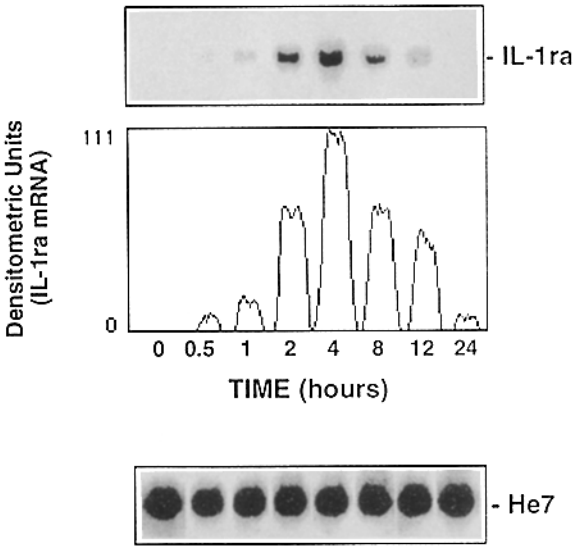

Figure 5. Kinetics of IL-4 induction of IL-1ra. Monocytes were incubated in suspension with IL-4 (1 ng/ml [Genzyme Corp.]) for 0–24 h during which RNA was isolated and probed for IL-1ra transcripts by Northern analysis, where He7 cDNA served as a housekeeping control. Resulting bands were analyzed by densitometry and results are expressed as arbitrary densitometric units.

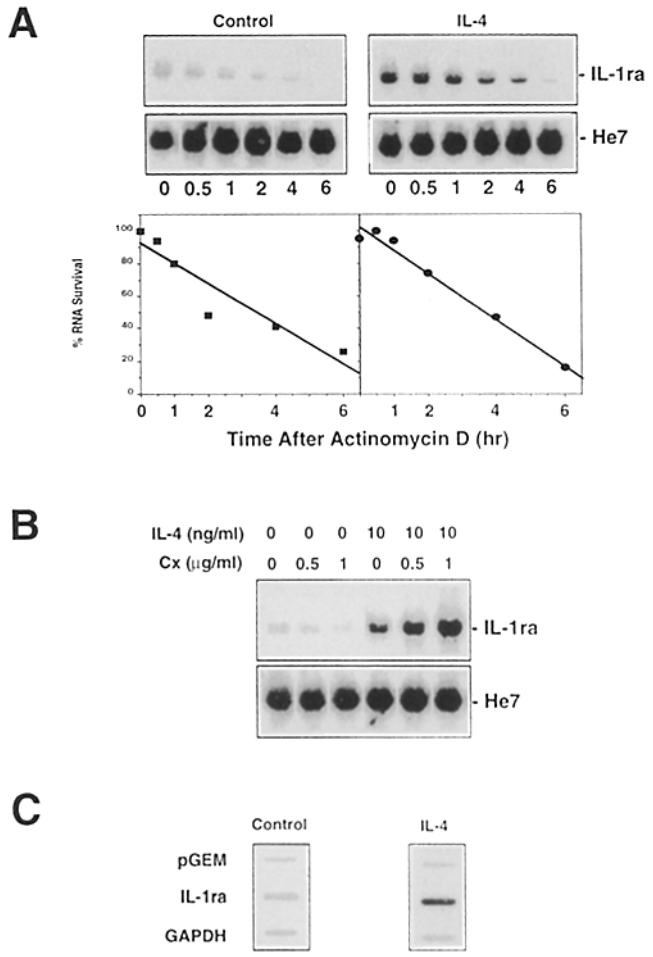

Figure 6. Regulation of IL-1ra mRNA by IL-4. (*A*) Monocytes were cultured in the presence or absence of IL-4 (10 ng/ml [Genzyme Corp.]) for 4 h after which actinomycin D (5 μg/ml) was added. At the indicated times, RNA was isolated for Northern blot analysis. The resulting RNA bands were analyzed densitometrically and the resulting values plotted against time. (*B*) Monocytes were cultured with or without IL-4 (10 ng/ml) in the presence or absence of Cx (0.5–1.0 μg/ml) for 4 h at 37°C, and RNA harvested for Northern analysis. (*C*) After monocytes (5×10^7) were cultured in the presence or absence of IL-4 (10 ng/ml) for 4 h, nuclei were isolated and a nuclear runoff assay performed. pGEM cDNA served as a vector control, whereas GAPDH cDNA served as a housekeeping control.

fixed on nitrocellulose filters. A threefold increase in the amount of synthesized transcripts for IL-1ra occurred after monocytes had been cultured with IL-4 (Fig. 6 *C*), consistent with a direct effect of IL-4 on the rate of transcription.

Discussion

Originally defined by its ability to promote B cell growth and IgE synthesis, continuing exploration of the role of IL-4 in host defense has revealed its significant ability to suppress numerous mononuclear phagocyte functions (6–10) and promote programmed cell death (25). In this study, we define yet another mechanism whereby IL-4 may suppress the immune response as shown in cancer patients receiving IL-4 therapy. In these patients, we demonstrate that systemically administered IL-4 selectively induces transcription and translation of IL-1ra, a competitive inhibitor of the multifunctional cytokine, IL-1. Induced circulating levels of IL-1ra may provide an immunosuppressive signal not inconsistent with the lack of augmented tumoricidal activity observed in these patients (26). Although LPS (24), TGF-β (27, 28), and GM-CSF (24, 29) also induce IL-1ra, each of these mediators also induces variable amounts of IL-1. As shown here and in recent studies (14, 30), IL-4 appears unique in its ability to selectively induce IL-1ra in resting monocytes without affecting IL-1, whereas in monocytes stimulated by LPS (15, 16, 30, and Fig. 4) or TGF-β (Wahl, S., G. Costa, H. Wong, and M. Brandes, manuscript submitted for publication), IL-4 not only augments IL-1ra, but it dramatically inhibits IL-1β expression. In the tumor patients receiving exogenous IL-4, a number of compromised monocyte functions have been ascribed to IL-4 coincident with the increased expression of IL-1ra (7), which document its immunosuppressive potential. Moreover, elevated endogenous levels of IL-4, as occur in visceral leishmaniasis (31), are also reflected by suppressed monocyte functions and defects in cell-mediated immunity.

There are several possible mechanisms whereby IL-4 may influence IL-1ra gene transcription and monocyte function. The IL-4–induced accumulation of IL-1ra message appeared not to be due only to a decreased rate of degradation, and by performing nuclear runoff experiments, IL-4 was shown to induce an increase in the rate of transcription. Furthermore, the ability of Cx to superinduce IL-4–mediated IL-1ra mRNA suggests that a regulatory protein may exist to repress transcription, possibly by interacting with a negative regulatory element in the promoter region of the IL-1ra gene (32). Although the region that is located 294 bp proximal of the promoter contains the critical sequences necessary for regulated expression of IL-1ra transcripts, it is unlikely that a putative labile protein repressor acts directly at the transcriptional level since Cx does not affect the production of IL-1ra mRNA in the absence of IL-4. It is conceivable that IL-4 interacts directly with a labile cytosolic regulatory molecule, and a depletion of this molecule may modulate IL-4 effects on the cell. Dissecting these pathways is crucial to defining the reciprocal effects of IL-4 on IL-1β and IL-1ra.

By competitively blocking IL-1 binding to the IL-1R and lacking any agonist effect, IL-1ra exerts a variety of antiinflammatory activities. For example, IL-1–mediated leukocyte adhesion to vascular endothelium is blocked by IL-1ra (33). The antagonist also reverses IL-1–induced production of PGE_2 and collagenase by fibroblasts, synoviocytes, and chondrocytes in culture (34, 35). Moreover, as an inhibitor of IL-1–induced degradation of proteoglycans and of glycosaminoglycan synthesis in cartilage explants (36), IL-1ra has been proffered as a potential therapeutic agent for arthritic lesions. Taking these in vitro observations a step further, IL-1ra has been administered in vivo and found to block IL-1–induced accumulation of leukocytes in inflamed joints (37, 38), and to reduce proteoglycan release from articular cartilage

(37). The demonstrated ability of IL-1ra to ameliorate arthritis in both the type II collagen model (39) and the bacterial cell wall model (40) has prompted clinical trials of this antagonist in rheumatoid arthritis (41).

Although IL-1ra is an important antiinflammatory agent, IL-4, which not only induces IL-1ra production, but also has numerous additional antiinflammatory effects, may prove a more effective inhibitor of chronic or aberrant immune function. In this regard, recent studies in bacterial cell wall–induced arthritis have documented that systemic administration of IL-4 does, in fact, increase expression of IL-1ra in the arthritic animals with a concomitant decrease in monocyte–macrophage functions, and suppression of joint swelling and tissue destruction (42; Allen, J., H. Wong, G. Costa, M. Bienkowski, and S. Wahl, manuscript submitted for publication). These observations are consistent with the induction of IL-1ra in cancer patients receiving exogenous IL-4 therapy in this study, and suggest that the use of systemically administered IL-4 may have important clinical significance as an antiinflammatory agent, especially in chronic inflammatory disease. Parenthetically, although no demonstrable antitumor effects were observed in the cancer patients treated with IL-4 alone (26), one patient with a chronic psoriatic condition improved clinically during the course of IL-4 treatment (Lotze, M., unpublished observation). Through the induction of IL-1ra and its emerging spectrum of monocyte inhibitory activities, IL-4 may prove to have important clinical applications.

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