ORIGINAL SCIENTIFIC PAPERS
CONSTITUTIVE mRNA AND PROTEIN PRODUCTION OF MACROPHAGE COLONY-STIMULATING FACTOR BUT NOT OF OTHER CYTOKINES BY SYNOVIAL FIBROBLASTS FROM RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS PATIENTS

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SUMMARY

This study analyses the mRNA and protein production and their regulation of macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8 and IL-6 by synovial fibroblasts obtained from patients with RA and OA. M-CSF was found to be produced constitutively as opposed to other cytokines. Stimulation of the cells with IL-1β caused a marked increase of GM-CSF, IL-8, IL-6 and as well as of M-CSF mRNA levels. In parallel, a time-dependent increase of M-CSF, GM-CSF, IL-8 and IL-6 protein production was observed. Among the cytokine mRNAs examined only that of M-CSF exhibited a pronounced stability in unstimulated synovial fibroblasts, whereas the other cytokines displayed short mRNA half-lives of 1–2 h. Induction by IL-1β markedly prolonged IL-8, IL-6 and GM-CSF mRNA half-lives to >8 h which indicates increased mRNA stability. These findings suggest that among the cytokines that are produced in the inflamed synovium M-CSF may be particularly important for sustaining long-term influx, activation and survival of mononuclear phagocytes. GM-CSF, IL-8 and IL-6, by contrast, may be more involved in more acute cellular responses.

KEY WORDS: Cytokines, mRNA stability, Synovial fibroblasts, Rheumatoid arthritis.

MACROPHAGE colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8 and IL-6 are representatives of a very complex cytokine network involved in the pathophysiology of RA. After activation by IL-1 and/or TNF-α synovial fibroblasts produce large amounts of these cytokines which account for a number of local and systemic manifestations of RA [1]. Fibroblast-derived cytokines interact in a synergistic or antagonistic fashion with cytokines released from macrophages, chondrocytes, endothelial cells and T cells. It is, however, difficult to appreciate the contribution of a single cytokine to the pathophysiological process leading to chronic synovitis and tissue destruction.

M-CSF augments functional activities and survival of monocytes and macrophages [2–4], whereas GM-CSF stimulates both neutrophils and macrophages [5,6]. IL-6 induces the final differentiation of B-lymphocytes into antibody-producing plasma cells thus contributing to local RF production and stimulates the release of major acute-phase proteins [7,8]. IL-8 is a potent neutrophil attractant and activator [9,10]. Therefore, these cytokines are of major importance for the type of cellular infiltration and the degree of local and systemic inflammation. In this study, we analysed the kinetics of mRNA and protein production as well as mRNA stability of M-CSF, GM-CSF, IL-8 and IL-6 in cultured synovial fibroblasts from RA and OA patients to examine possible differences of regulation

and contribution to either acute or chronic inflammatory processes.

MATERIAL AND METHODS

Patients

Synovial tissue was obtained from seven patients with active RA (five females, two male) and four patients with OA (two female, two male) undergoing total prosthetic joint replacement of the knee. All patients received only NSAIDs up to 1 day before orthopaedic surgery. The RA patients were seropositive and fulfilled the revised ARA criteria for RA [11].

Synovial fibroblast isolation and culture

Cells were isolated from synovial tissue and collagenase digestion was performed as previously described [12]. Synovial cells were plated into 75 cm² culture flasks (Becton & Dickinson, Basel, Switzerland) in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin and 10% heat-inactivated fetal calf serum (FCS) in a humidified 5% CO_2 atmosphere at 37°C. After 1 day in culture non-adherent cells were removed. In order to obtain homogenous populations of fibroblast-like cells synoviocytes were passaged four times by brief trypsinization and recultured in medium. Synovial fibroblast cultures were found to be free of contaminating cells (lymphoid and monocytic cells) as assessed by morphology (Wright–Giemsa staining), non-specific esterase staining [13] and specific immunostaining with anti-CD3, and CD14 mAbs (Becton & Dickinson, Basel, Switzerland). In most experiments,

cells (1–1.5 × 10⁶) were incubated in RPMI 1640 supplemented with 1% pasteurized plasma albumin solution (PPL, Swiss Red Cross, Bern, Switzerland) in the presence or absence of rhuIL-1β.

Assessment of cytokine protein release

M-CSF was determined by a radioimmunoassay that used rhuM-CSF as the standard, radioiodinated rhuM-CSF as tracer, and a rabbit antiserum to rhuM-CSF [14]. The detection limit was 5 U/ml. GM-CSF was determined using a sandwich immunoassay with a cocktail of three epitope-mapped monoclonal antibodies (mAbs) [15]. The detection limit was 0.2 ng/ml. IL-8 was determined by a solid phase double-ligand ELISA method [16] with a detection limit of 50 pg/ml, and IL-6 by a commercially available rhuIL-6 immunoassay (Research and Diagnostic Systems, Minneapolis, MN, USA) with a detection limit of 4 pg/ml.

Assessment of RNA levels by Northern blotting
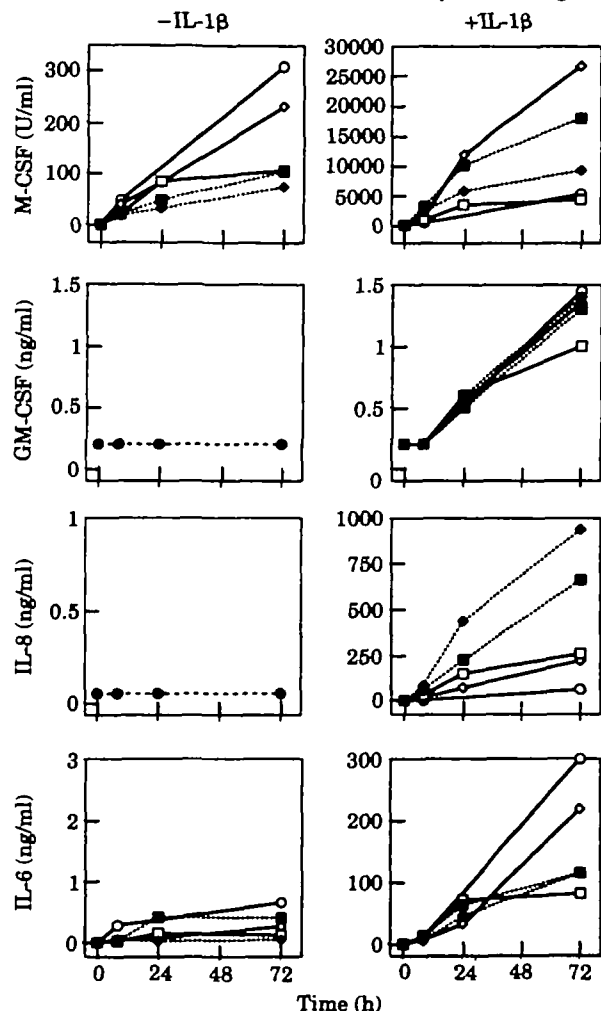
Total cellular RNA was extracted by the acid guani-

FIG. 1.—Constitutive and IL-1β-stimulated secretion of M-CSF, GM-CSF, IL-8 and IL-6. Synovial fibroblasts from three RA (open symbols) and two OA (closed symbols) patients were cultured for up to 72 h in the absence and presence of IL-1β (10 ng/ml). Levels of GM-CSF and IL-8 were below detection limit of the assay in unstimulated cells (●– – –●). Results represent the mean of duplicate determinations for each patient.

dium thiocyanate phenol-chloroform method [17]. Equal amounts of RNA were size separated by an agarose formaldehyde gel (1% wt/vol) and transferred to a nylon membrane (Hybond-N, Amersham, UK). Hybridization with random primer (³²p)-labelled probes (1×10⁶ cpm/ml hybridization solution) was for 16 to 24 h at 42°C [18,19]. Filters were washed to a final stringency of 0.1% SSC at 65°C, and exposed for 6 h to 4 days at –70°C to XM-films (3M, Trimax, Ferrania, Italy). Autoradiographs were scanned by laser densitometry (LKB 2202 Ultrascan, Bromma, Sweden).

DNA probes

Purified inserts were used as DNA probes. The human M-CSF cDNA was purified from the pcCSF-12 plasmid (1.6 kb, Xhol-EcoRI) [20], IL-6 cDNA was from plasmid pXM (0.5 kb, Pst1) (Genetics Institute, Cambridge, MA, USA), and IL-8 cDNA was derived from plasmid p (NAP) 6T3 (0.85 kb, BamHI) [21]. The human GM-CSF cDNA probe (0.8 kb, EcoRI-BamHI) was derived from plasmid pCSF-1 [22].

Reagents

rhuIL-1β was a kind gift from Dr Vosbeck, Ciba-Geigy, Basel, Switzerland and was used at a final concentration of 10 ng/ml which gave optimal fibroblast stimulation in prior dose–response experiments. Actinomycin D (Fluka, Buchs, Switzerland) was dissolved in DMSO at 5 mg/ml. The final concentration of DMSO never exceeded 0.12% vol/vol which had no effect on cytokine mRNA levels in synovial fibroblasts. The endotoxin content of reagents stocks was less than 6 pg/ml (limulus amebocyte lysate assay).

RESULTS

Release of M-CSF, GM-CSF, IL-6 and IL-8

Figure 1 shows the release of the four cytokines by RA and OA synovial fibroblasts in the presence and absence of IL-1β. In all samples a constitutive secretion was observed only for M-CSF, which was more pronounced in RA synovial fibroblasts than in OA cells. Only small amounts of IL-6 were detected under these conditions, and the values for GM-CSF and IL-8 were below the detection limit. Stimulation with IL-1β led to a marked, time-dependent increase of production of all four cytokines in both RA and OA synovial cells with maximal release after 72 h of incubation.

Steady-state mRNA levels of M-CSF, GM-CSF, IL-8 and IL-6

In the same set of experiments mRNA levels of the four cytokines were examined in synovial fibroblasts from OA and RA patients (Figs 2 and 3). As shown by the blots and the corresponding quantitative densitometry, constitutive M-CSF mRNA production was observed by both OA [Figs 2 (a) and (b)] and RA [Figs 3 (a) and (b)] synovial fibroblasts during the whole culture period of 72 h whereas mRNA levels for the other cytokines were barely detectable. When stimulated with IL-1β, GM-CSF, IL-8 and IL-6 mRNA levels were already detectable after 2 h of incubation and maximal
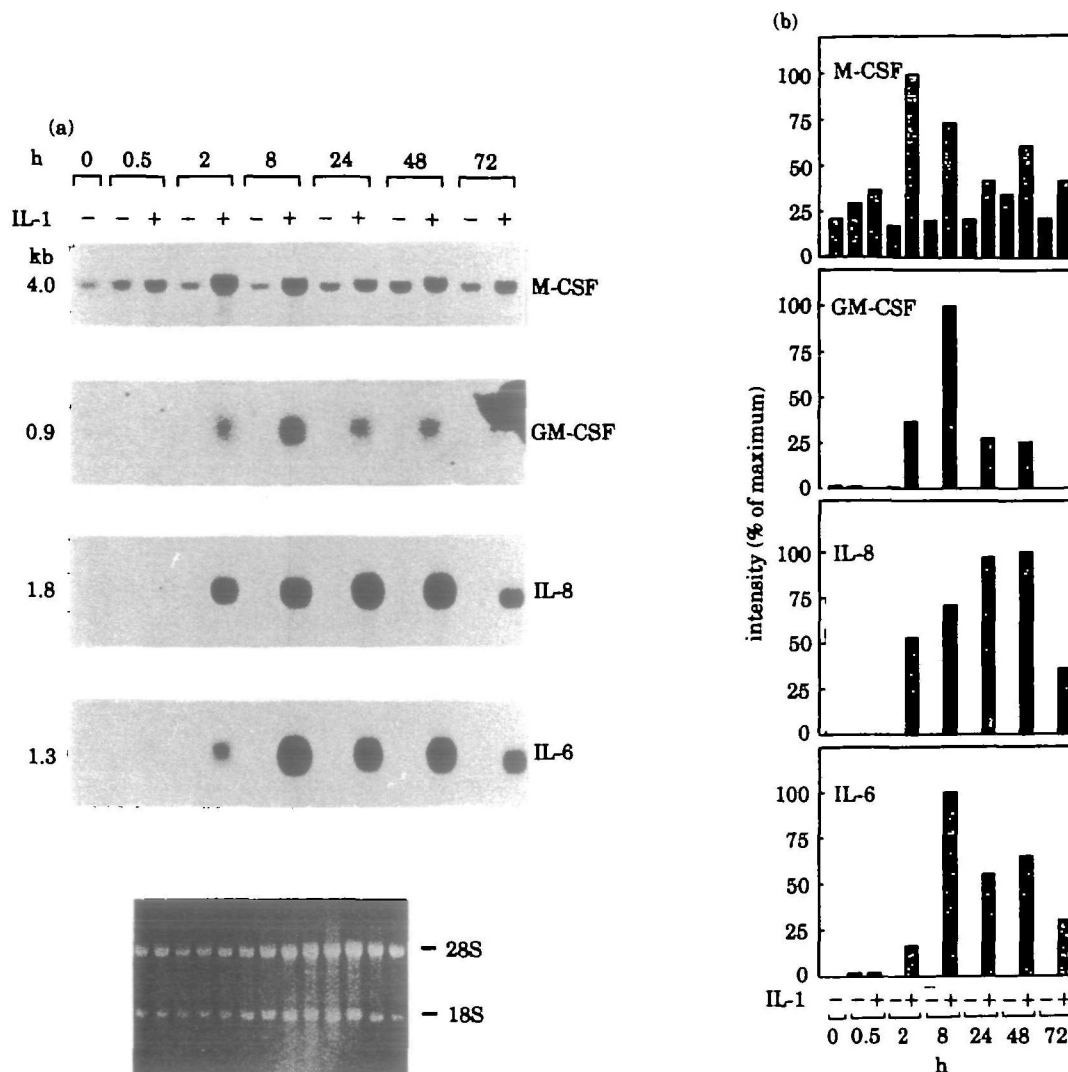
FIG. 2.—mRNA expression of M-CSF, GM-CSF, IL-8 and IL-6 by synovial fibroblasts from a representative OA patient. The cells were cultured with (+) or without (−) IL-β (10 ng/ml) for 0 to 72 h. 10 μg of RNA were loaded per lane. (a) The Northern blot was hybridized sequentially with M-CSF, GM,-CSF, IL-8 and IL-6 cDNA probes. The small bottom panel shows the ethidium bromide stained Northern gel with 28s and 18s rRNA, demonstrating equivalent RNA loading per lane. Transcript sizes are indicated in kb. (b) Evaluation of the autoradiographs by laser densitometry. The 100% values refer to cells with maximal mRNA levels of the respective cyokines.

levels were observed after 8 h. Expression of GM-CSF mRNA declined after 24 h in both synovial fibroblasts from OA and RA patients, whereas mRNA levels for M-CSF, IL-8 and IL-6 mRNAs remained high.

Cytokine mRNA stability

To elucidate whether the differential expression of the four cytokines may be due to different mRNA stability, experiments were performed with actinomycin D to determine the mRNA half-lives. RA cells were cultured for 10 h [short-term cultures; Figs 4 (a) and (b)] and 7 days [long-term cultures; Figs 5 (a) and (b)] in RPMI 1640 and 10% FCS with or without IL-1β. Subsequently the cells were exposed to actinomycin D (6 μg/ml) for 1 to 8 h. In short-term cultures the half-live of M-CSF mRNA was >8 h in both unstimulated and stimulated cells. In contrast, the basal mRNA levels of GM-CSF, IL-8 and IL-6, though detectable at low levels, declined with half-lives of approximately 1–2 h.

Stimulation with IL-1β resulted in an increase of their mRNA half-lives to >8 h. Similar results were obtained in long-term cultures [Figs 5 (a) and (b)]. Under these conditions the half-life of M-CSF mRNA remained >8 h, whereas the decay of the basal mRNA of IL-8 and IL-6 corresponded to short half-lives of about 1 h. No steady-state basal GM-CSF mRNA levels were detectable in long-term cultures. Again, following stimulation with IL-1β, mRNA levels of GM-CSF, IL-8 and IL-6 were stable with half-lives of >8 h. Similar observations were obtained in five independent experiments using cells derived from three RA and two OA patients.

DISCUSSION

In this study we examined production and regulation of inflammatory cytokines at both RNA and protein levels in fibroblast cultures derived from synovial tissue of RA and OA patients. We found that synovial fibro-
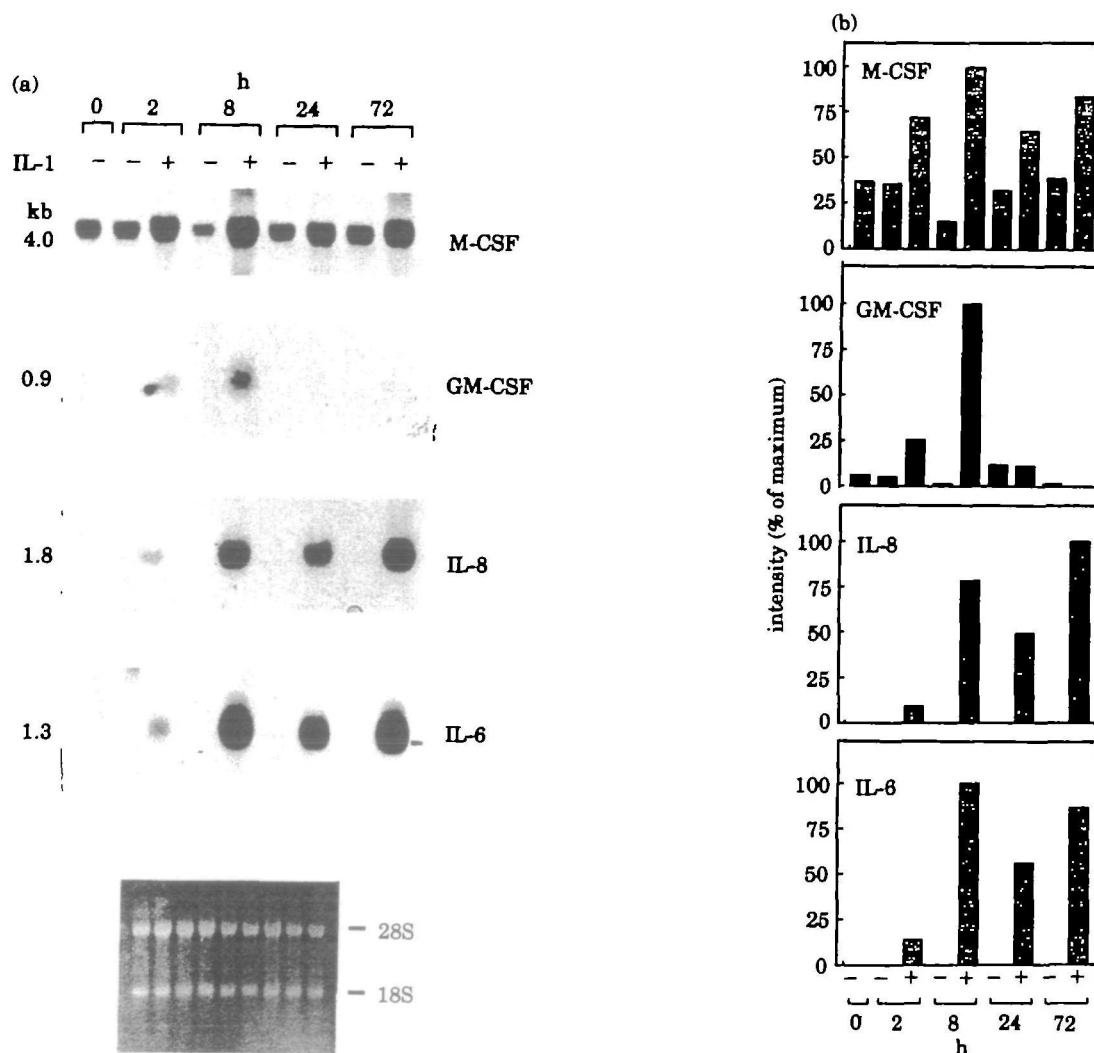
Fig. 3.—mRNA expression of M-CSF, GM-CSF, IL-8 and IL-6 by synovial fibroblasts from a representative RA patient. The cells were cultured with (+) or without (−) IL-1β (10 ng/ml) for 0 to 72 h. 10 μg of RNA were loaded per lane. (a) The Northern blot was hybridized sequentially with M-CSF, GM-CSF, IL-8 and IL-6 cDNA probes. The small bottom panel shows the ethidium bromide stained Northern gel with 28s and 18s rRNA, demonstrating equivalent RNA loading per lane. Transcript sizes are indicated in kb. (b) Evaluation of the autoradiographs by laser densitometry. The 100% values refer to cells with maximal mRNA levels of the respective cytokines.

blasts produce M-CSF mRNA and protein. M-CSF protein was constitutively released from RA and OA synovial fibroblasts as opposed to GM-CSF, IL-8 and IL-6 whose mRNA and protein production only increased after IL-1β stimulation. M-CSF mRNA production was constant over time, thus giving rise to the marked and prolonged constitutive M-CSF protein release. The different expression pattern of M-CSF as compared with that of GM-CSF, IL-8 and IL-6 in synovial fibroblasts is in keeping with studies using fibroblasts of other tissues. For example, resting human embryonic lung fibroblasts did not produce spontaneously GM-CSF, IL-8 and IL-6, but constitutively produced M-CSF mRNA and protein [23] and a similar pattern of M-CSF gene expression was found in human dermal fibroblasts [24]. Increased CSF protein production has also been seen in synovial cells [25–29]. Constitutive M-CSF production is not unique to diseased synovial fibroblasts but may be higher in inflammatory

disease processes where marked cell activation occurs in vivo.

To elucidate in more detail the disparate expression pattern of M-CSF, we compared the mRNA stability of the four cytokines. Our experiments revealed that M-CSF mRNA is very stable with a half-life of >8 h in both unstimulated and IL-1β-exposed cells of RA and OA patients. Although there were no big differences in M-CSF mRNA expression of unstimulated and IL-1β stimulated synovial fibroblasts, activated cells exhibited more than a 100-fold release of M-CSF protein as compared with resting fibroblasts. This discrepancy suggests that M-CSF production is not only regulated at the level of mRNA stability but also at the translational level. One explanation for the different expression pattern of M-CSF might be that rapidly up- and down-regulated cytokines such as GM-CSF, IL-8 and IL-6 possess repeated AUUUA sequences in the 3′ untranslated region of the mRNAs whilst M-CSF
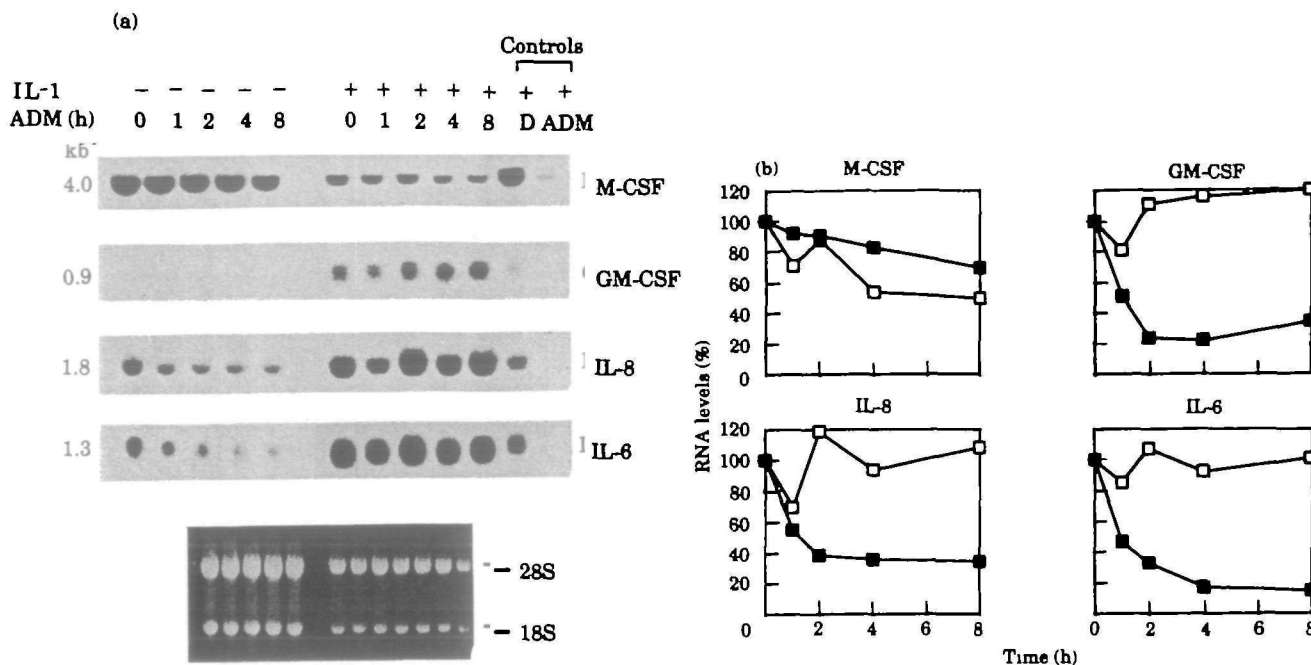
FIG. 4.—Cytokine mRNA stability in unstimulated and short-term stimulated RA synovial fibroblasts. (a) Cells were cultured with (+) or without (−) IL-1β (10 ng/ml) for 10 h and without medium exchange for subsequent 0 to 8 h with actinomycin D (6 μg/ml). 15 μg of RNA per lane were loaded from unstimulated cells and 7.5 μg from IL-1β-stimulated cells. Controls include cells treated simultaneously for 8 h with IL-1 DMSO (D) and cells treated simultaneously for 8 h with IL-1 and actinomycin D (ADM). The Northern blot was hybridized sequentially with M-CSF, GM-CSF, IL-8 and IL-6 cDNA probes. The small bottom panel shows the ethidium bromide stained Northern gel with 28s and 18s rRNA, demonstrating equivalent RNA loading per lane. Transcript sizes are indicated as kb. (b) Relative mRNA levels of M-CSF, GM-CSF, IL-8 and IL-6 in unstimulated (■) and IL-1β-stimulated (□) RA synovial fibroblasts. Although no signal for GM-CSF mRNA is apparent on the presented autoradiograph a longer exposure allowed the quantification by densitometry reading. The 100% values refer to cytokine mRNA levels of cells stimulated for 10 h with IL-1β.

mRNA does not [30]. Earlier studies have demonstrated that these sequences are critical for RNA stability [31]. In contrast to M-CSF, mRNAs of GM-CSF, IL-8 and IL-6 were short-lived in unstimulated synoviocytes and became more stable in the presence of IL-1β. It is noteworthy that in particular GM-CSF mRNA markedly declined after having reached its maximal production at 8 h of IL-1β incubation despite the continued presence of the stimulus. Since no change in stability of GM-CSF mRNA transcripts was observed, this decrease may be due to reduced gene transcription.

In most experiments synovial fibroblasts were studied in a medium containing only 1% human albumin to avoid cytokine induction by growth factors or other agents that may be present in serum. This might explain why our results contrast with previous studies which showed constitutive release of TNF-α, IL-1β, GM-CSF and IL-6 from synoviocytes when cultures were supplemented with 10% FCS [32,33].

The question arises as to the *in vivo* significance of this particular M-CSF expression and regulation. Because M-CSF stimulates IL-1β gene transcription, enhances mRNA stability and protein secretion in human monocytes [34] and primes these cells for subsequent TNF-α induction by other stimuli [35], the observed constitutive production by M-CSF by synovial fibroblasts could play a critical role in the autocrine/paracrine inflammatory cytokine network

leading to persistent synovitis. Monocytes-macrophages, after appropriate stimulation, can synthesize M-CSF [36,37]. M-CSF again has the potential to feedback on the same or adjacent mononuclear phagocyte to produce other cytokines as for example IL-1 or TNF-α.

These cytokines then stimulate neighbouring fibroblasts, endothelial cells and chondrocytes to produce CSFs. M-CSF so formed can feedback again on the tissue macrophage. Consequently, a strong and prolonged constitutive M-CSF production by synovial fibroblasts as found in this study, is likely to play a critical role in sustaining a continuous influx of monocytes-macrophages from their progenitors as well as in favouring their survival at sites of synovitis. Since the expression of M-CSF mRNA in fibroblasts as opposed to that of GM-CSF, IL-8 and IL-6, is not inhibited by glucocorticoids [23], M-CSF may be an important target molecule for novel anti-cytokine therapy of chronic inflammation.
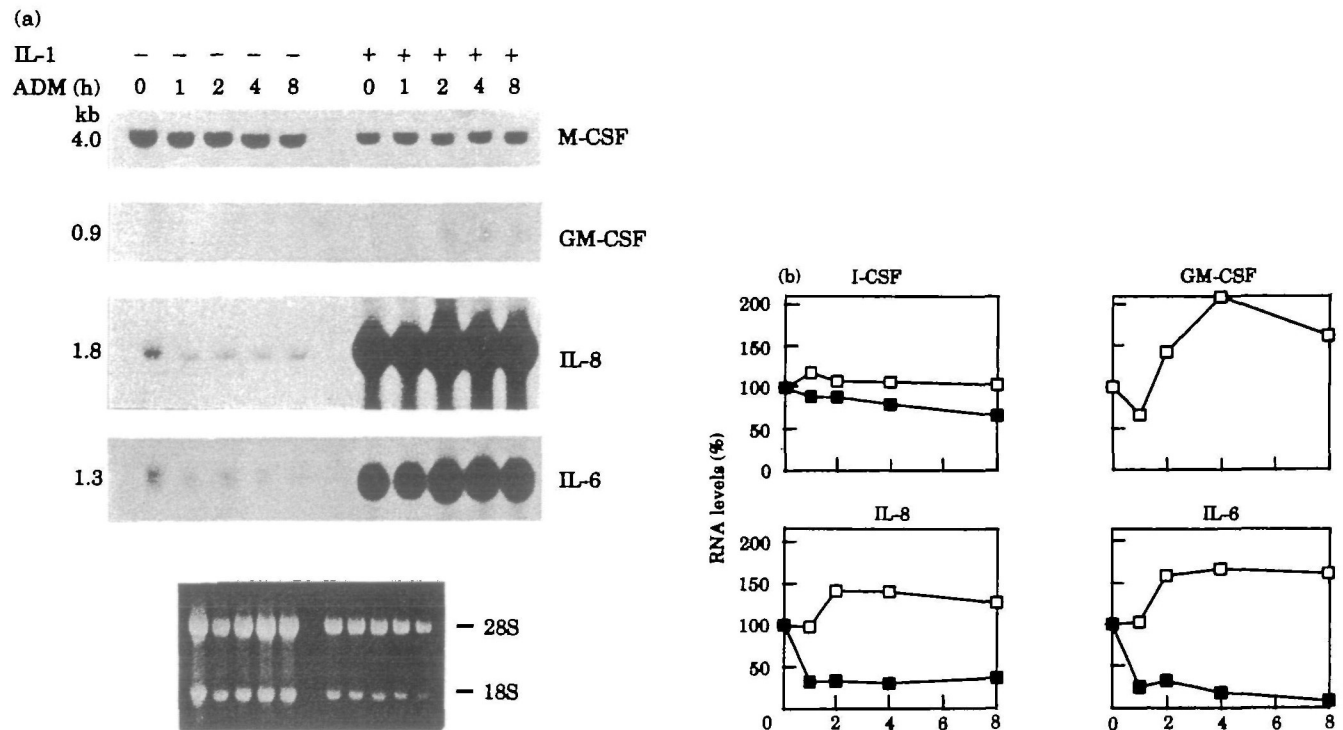
(a)

FIG. 5.—Cytokine mRNA stability in unstimulated and long-term stimulated RA synovial fibroblasts. (a) Cells were incubated with (+) or without (−) IL-1β (10 ng/ml) for 7 days and without medium exchange for subsequent 0 to 8 h with actinomycin D (6 μg/ml). 15 μg of RNA per lane were loaded from unstimulated cells and 7.5 μg from IL-1β-stimulated cells. The Northern blot was hybridized sequentially with M-CSF, GM-CSF, IL-8 and IL-6 cDNA. The small bottom panel shows the ethidium bromide stained Northern gel with 28S and 18S rRNA, demonstrating equivalent RNA loading per lane. Transcript sizes are indicated as kb. (b) Relative mRNA levels of M-CSF, GM-CSF, IL-8 and IL-6 in unstimulated (■) and IL-1β-stimulated (□) RA synovial fibroblasts. Even after a long exposure of autoradiographs no GM-CSF mRNA was detectable in unstimulated cells. Intensities of hybridization signals were determined by densitometry reading and the 100% values refer to cytokine mRNA levels of cells stimulated for 7 days with IL-1β.

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