Cytokines in Chronic Inflammatory Arthritis. V. Mutual Antagonism between Interferon-Gamma and Tumor Necrosis Factor-Alpha on HLA-DR Expression, Proliferation, Collagenase Production, and Granulocyte Macrophage Colony-stimulating Factor Production by Rheumatoid Arthritis Synoviocytes

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Abstract

The effects of a broad array of cytokines, individually and in combination, were determined on separate functions (proliferation, collagenase production, and granulocyte macrophage colony-stimulating factor [GM-CSF] production) and phenotype (expression of class II MHC antigens) of cultured fibroblast-like RA synoviocytes. The following recombinant cytokines were used: IL-1beta, IL-2, IL-3, IL-4, IFN-gamma, tumor necrosis factor (TNF)-alpha, GM-CSF, and macrophage colony-stimulating factor (M-CSF). Only IFN-gamma induced HLA-DR (but not HLA-DQ) expression. TNF-alpha inhibited IFN-gamma-mediated HLA-DR expression ($46.7\pm4.1\%$ inhibition) and HLA-DR mRNA accumulation. This inhibitory effect was also observed in osteoarthritis synoviocytes. Only TNF-alpha and IL-1 increased synoviocyte proliferation (stimulation index 3.60 ± 1.03 and 2.31 ± 0.46, respectively). IFN-gamma (but none of the other cytokines) inhibited TNF-alpha-induced proliferation ($70\pm14\%$ inhibition) without affecting the activity of IL-1. Only IL-1beta and TNF-alpha induced collagenase production (from <0.10 U/ml to 1.10 ± 0.15 and 0.72 ± 0.24, respectively). IFN-gamma decreased TNF-alpha-mediated collagenase production ($69\pm19\%$ inhibition) and GM-CSF production but had no effect on the action of IL-1. These data demonstrate mutual antagonism between IFN-gamma and TNF-alpha on fibroblast-like synoviocytes and suggest a novel homeostatic control mechanism that might be defective in RA where very little IFN-gamma is produced. (*J. Clin. Invest.* 1990. 86:1790–1798.) Key words: cytokine • interferon-gamma • tumor necrosis factor-alpha • synoviocyte • rheumatoid arthritis

Introduction

Synovial lining hyperplasia and destruction of extracellular matrix are characteristic histopathological findings in rheumatoid arthritis (1, 2). These phenomena are, in part, a consequence of increased numbers of fibroblast-like (type B) synoviocytes with a concomitant increase in their secretion of mediators involved in matrix destruction, such as prostaglandins and collagenase (3–6). The synovial cytokine milieu in the inflamed joint is thought to be influential in these changes. This notion is derived from the fact that cytokines induce a number of functional alterations in cultured synoviocytes that mimic those observed in the inflamed synovium. For instance, IL-1, tumor necrosis factor (TNF)-alpha,[1] platelet-derived growth factor, and basic fibroblast growth factor, each of which is present in RA synovial fluid, (7–12) increase cultured synoviocyte proliferation (13–17) and IL-1 and TNF-alpha can stimulate the synthesis of collagenase (18, 19) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Alvaro-Gracia, J. M., N. J. Zvaifler, and G. S. Firestein, submitted for publication) by synoviocytes. Also, cytokines are likely important in determining the surface phenotype of synoviocytes, particularly the expression of class II major histocompatibility markers. GM-CSF is a major inducer of HLA-DR expression on macrophage-like (type A) synoviocytes (20) and IFN-gamma has been shown to induce HLA-DR on cultured type B synoviocytes (21, 22).

Most studies on synovial cells have been performed to determine the effects of individual cytokines. However, it has become increasingly clear that these factors do not function in isolation; rather, the synovium is bathed in a mixture in which specific interactions might be important. In order to understand the role of cytokines in chronic arthritis better, we have studied the effects of a broad array of individual factors on separate functions (proliferation, collagenase production, and GM-CSF production) and phenotype (expression of class II MHC antigens) of cultured synoviocytes. We then analyzed their effects when used in combinations. As expected, IL-1beta and TNF-alpha enhanced each other's effect on synoviocyte proliferation and collagenase production. The most striking finding, however, was mutual antagonism between TNF-alpha and IFN-gamma; TNF-alpha inhibited IFN-gamma-mediated HLA-DR induction on cultured synoviocytes while IFN-gamma blocked TNF-alpha-induced proliferation and collagenase production. IFN-gamma also inhibited TNF-alpha-induced GM-CSF secretion. These data suggest a novel homeostatic control mechanism in the synovium that might be defective in RA.

Methods

Synoviocyte culture. Synovial tissues (ST) were obtained from patients with RA and osteoarthritis (OA) undergoing total hip or knee replacement surgery. Synoviocytes were isolated by collagenase digestion as previously described (23). Briefly, the tissues were minced and incu-

1. *Abbreviations used in this paper:* GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage CSF; MFC, mean fluorescence channel; OA, osteoarthritis; ST, synovial tissue; TNF, tumor necrosis factor.

bated with 2 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY) for 2 h at 37°C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% FCS (Gibco; endotoxin content < 0.006 ng/ml), penicillin, streptomycin, and L-glutamine (medium) in a humidified 5% CO_2 atmosphere. After overnight culture, nonadherent cells were removed and adherent cells were cultivated in DMEM plus 10% FCS. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes were used from passages three through seven in these experiments, during which time they were a homogeneous population of fibroblast-like synoviocytes (< 1% CD11b positive, $FC\gamma RII$, < 1% phagocytic, and < 1% receptor-positive).

Reagents. Recombinant hIL-1 beta (sp act 5×10^8 U/mg, purity > 95%) was purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant hIFN-gamma (sp act 2×10^7 U/mg, purity > 98%) and hGM-CSF (sp act 10^8 U/mg, purity > 95%) were a gift from Amgen Biologicals. Recombinant hIL-3 (sp act 10^8 CFU/mg, purity > 95%) was purchased from Genzyme Corp. (Cambridge, MA). Recombinant hIL-4 (sp act 10^8 U/mg, purity > 95%), was obtained from Immunex Corp. (Seattle, WA). Recombinant hIL-6 (sp act 5×10^9 U/mg, purity > 95%) was a gift from Dr. Martin Lotz (U.C.S.D. Medical Center, San Diego, CA). Recombinant hIL-2 (sp act 3×10^6 U/mg, purity > 95%) was obtained from Cetus Corp. (Emeryville, CA) and recombinant hM-CSF (CSF-1) (sp act 7×10^7 U/mg, purity > 95%) was a gift from Dr. R. Halenbeck, Cetus Corp. Recombinant hTNF-alpha (sp act 5×10^7 U/mg, purity > 95%) was provided by Genentech Inc. (South San Francisco, CA). In each case, the final concentration of LPS in culture after dilution of recombinant cytokine was < 0.005 ng/ml. The maximum concentrations of cytokines used were generally determined from standard biological assays (e.g., IL-2 in cytotoxic T lymphocyte line (CTLL) assay, GM-CSF in HLA-DR induction assay on monocytes, M-CSF in CFU assay using murine bone marrow, etc.). The range of concentrations tested in the various assays were: IL-1beta (1–100 U/ml), IL-2 (1–100 ng/ml), IL-3 (1–100 U/ml), IL-4 (1–100 U/ml), TNF-alpha (1–100 ng/ml), IFN-gamma (1–100 U/ml), GM-CSF (8–800 U/ml), and M-CSF (10–1,000 U/ml). Polymyxin B (7,900 U/mg) was purchased from Sigma Chemical Co. (St. Louis, MO).

HLA-DR and DQ surface expression. HLA-DR and DQ expression was assayed by immunofluorescence and FACS analysis. HLA-DR was detected with the MAb SC2 (IgG_2, kindly provided by Dr. Robert Fox, Scripps Clinic Research Foundation, La Jolla, CA); HLA-DQ was detected with the MAb Leu 10 (IgG_1, Becton Dickinson Immunocytometry Systems, Mountain View, CA). Synoviocytes ($1-2 \times 10^5$) were cultured in 6-well plastic dishes (Costar Data Packaging Corp., Cambridge, MA) for 3 d in DMEM-10% FCS and cytokines. Cells were harvested by brief trypsinization and stained with the corresponding MAb as described (24). The brief trypsinization does not affect detection of surface HLA-DR or DQ (data not shown). Phycoerythrin-conjugated Fab_2 anti–mouse IgG (Tago Inc., Burlingame, CA) was used as secondary antibody instead of FITC-conjugated to avoid problems with the green autofluorescence exhibited by synoviocytes. FACS analysis was performed in a cytofluorograph IIs (Ortho Diagnostic Systems Inc., Raritan, NJ). 5,000 cells were analyzed from each sample adjusting the fluorescence gain so that approximately 5% of the cells of the sample with greatest fluorescence were positive in the highest fluorescence channel. The results are presented as absolute or relative mean fluorescence channel (MFC) of total cells. The relative MFC = absolute $MFC_{cytokine}$/absolute MFC_{medium}.

mRNA analysis. Northern blot analysis was performed as previously described (20). Briefly, total cytoplasmic RNA was isolated from $1-2 \times 10^6$ synoviocytes cultured for 48 h with cytokines using the acid guanidine phenol chloroform protocol (25). 20 μg of RNA were run on a 1.2% agarose-formaldehyde gel and blotted onto a nylon membrane (Schleicher & Schuell, Inc., Keene, NH). The HLA-DR beta chain probe (kindly provided by Dr. Per Peterson, Scripps Clinic Research Foundation, La Jolla, CA) was radiolabeled with [^{32}P]dCTP

(Amersham Corp., Arlington Heights, IL) by the random primer method (Bethesda Research Laboratories, Gaithersburg, MD) and the filter was hybridized overnight at 42°C. The filter was washed and exposed with Kodak X-AR film at −20°C and an intensifying screen. Ethidium bromide staining of ribosomal RNA was used as control for the amount of RNA loaded in the different lanes.

Proliferation. Subconfluent synoviocytes were passed into flat-bottom 96-well dishes (Linbro, McClean, VA) at 10^4 cells/well. After overnight incubation, cells were cultured in DMEM containing 10% FCS and cytokines for 3 d. 1 μCi of [^{3}H]thymidine (Amersham) was then added to the cultures and the cells were incubated for 24 more h. At the end of the incubation period, cells were washed with PBS, detached with 0.1% trypsin-0.04% EDTA, and harvested onto glass-fiber filters using a microharvesting device (Otto Hiller Co., Madison, WI). Incorporated radioactivity was measured using a liquid scintillation counter (Southern Biotechnology Associates, Birmingham, AL). The results are shown either as total cpm or as stimulation index $= cpm_{cytokine}/cpm_{medium}$. In some experiments, cells were starved in 1% FCS for 3 d and the proliferation assay was subsequently performed in this medium.

Collagenase activity. Synoviocytes were plated in 24-well dishes at 1.5×10^5 cells/well in DMEM-10% FCS. After overnight incubation the medium was replaced with 0.4 ml of 1% FCS DMEM plus cytokine. Supernatants were collected after 3 d of culture and stored at −70°C until assayed. Collagenase activity was measured in these supernatants after treatment with trypsin (Sigma) 100 μg/ml for 20 min followed by a fivefold excess of soybean trypsin inhibitor to activate latent collagenase. An indirect ELISA, according to the method of Yoshioka et al. (26), was used. Briefly, flat-bottom 96-well dishes (Linbro) were coated with type I calf skin collagen (Sigma) for a minimum of 7 d at 4°C. The plates were washed with PBS and samples were diluted 1 to 2 in a Tris-HCl buffer (26) and incubated for 1 h at 37°C. The undigested collagen was detected with affinity-purified goat anti–human type I collagen antibody (Southern Biotechnology) followed by an alkaline phosphatase-labeled affinity-purified rabbit anti–goat IgG antibody (Sigma). *Para*-nitrophenyl-phosphate (Sigma) 1 mg/ml was used as substrate and the ODs were determined at 405 nm in an ELISA reader (Bio-Rad Laboratories, Richmond, CA).

GM-CSF assay. GM-CSF was assayed by Dr. Kenneth Kaushansky and Dr. Christopher B. Brown (University of Washington, Seattle, WA) using a specific solid-phase ELISA as previously described (27).

Statistics. Statistical analysis was performed using the Student's *t* test.

Results

HLA-DR and HLA-DQ expression on cultured synoviocytes

Effect of individual cytokines. Initial experiments compared the effect of several individual cytokines on HLA-DR surface expression on synoviocytes (third to seventh passage) after 3 d incubation. The results were analyzed by FACS. Unstimulated cultured synoviocytes had no detectable class II MHC antigens (DR or DQ) on their surface. As has been noted by others (21, 22), IFN-gamma was a potent inducer of HLA-DR. None of the other cytokines tested altered DR expression, including several mediators that induce class II MHC antigens on other cell types (e.g., TNF-alpha, GM-CSF, and IL-4) (see Fig. 1). None of the cytokines (including IFN-gamma) induced HLA-DQ expression (data not shown).

Cytokine interactions. The ability of other cytokines to modulate the HLA-DR-inducing effect of IFN-gamma was then studied. In these experiments, the other individual cytokines were added to synoviocytes simultaneously with IFN-gamma. IL-1beta, IL-2, IL-3, IL-4, GM-CSF, and M-CSF had
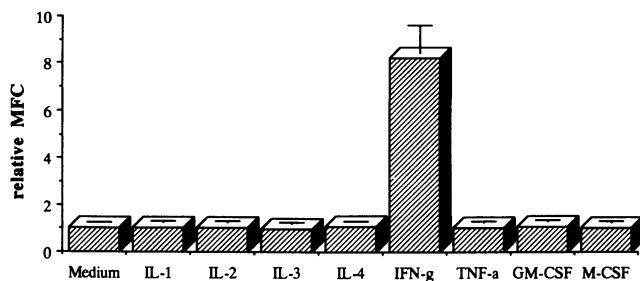
Figure 1. HLA-DR induction by cytokines on RA type B synoviocytes. Third to seventh passage synoviocytes were incubated for 72 h in DMEM-10% FCS and cytokines (IL-1beta 100 U/ml; IL-2 100 ng/ml; IL-3 100 U/ml; IL-4 100 U/ml; IFN-gamma 100 U/ml; TNF-alpha 100 ng/ml; GM-CSF 800 U/ml; and M-CSF 1,000 U/ml). Cells were analyzed for HLA-DR expression by FACS. Results are shown as relative MFC±SEM (see Methods); $n = 9$ for medium, IFN-gamma and TNF-alpha; $n = 5$ for IL-4 and GM-CSF; $n = 3$ for other cytokines.

no effect on the IFN-gamma-induced HLA-DR expression (data not shown). However, TNF-alpha significantly inhibited the IFN-gamma-mediated HLA-DR expression on synoviocytes (49.3±5.1% inhibition, $n = 6$, $P < 0.001$) (see Fig. 2). This inhibitory effect was not due to contaminating endotoxin since no difference was detected in experiments performed in the presence of polymyxin B (16 μg/ml) (data not shown). To determine if the effects of IFN-gamma and TNF-alpha on HLA-DR expression were unique to RA synoviocytes, the response of cells isolated from rheumatoid ($n = 6$) and osteoarthritic ($n = 5$) synovia were compared. Fig. 2 shows that both groups behaved similarly. A representative dose response for the effect of TNF-alpha on various concentrations of IFN-gamma is shown in Fig. 3 *A*. The inhibitory effect was not simply due to delayed kinetics of DR expression since inhibition was observed within 1 d and persisted for at least 6 d (see Fig. 3 *B*). Because of recent interest in the role of GM-CSF on synovial macrophage HLA-DR expression (20, 28), we tested GM-CSF in combination with several other cytokines. Neither GM-CSF alone nor in combination with TNF-alpha, M-CSF, or IL-4 induced HLA-DR expression on synoviocytes (data not shown).
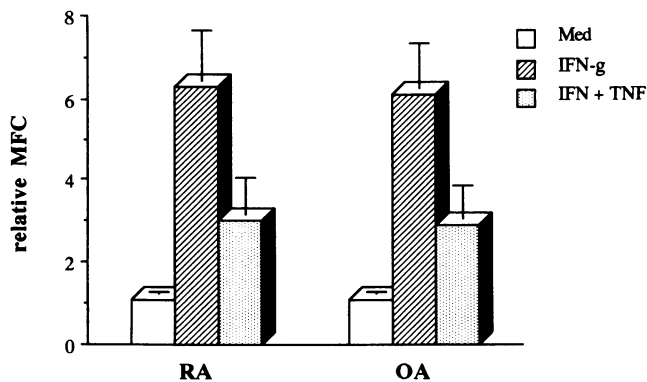

Figure 2. Inhibitory effect of TNF-alpha (50 ng/ml) on IFN-gamma (100 U/ml) induced synoviocyte HLA-DR expression. RA ($n = 6$) and OA ($n = 5$) synoviocytes (third to seventh passage) were incubated for 72 h in medium with or without cytokines.
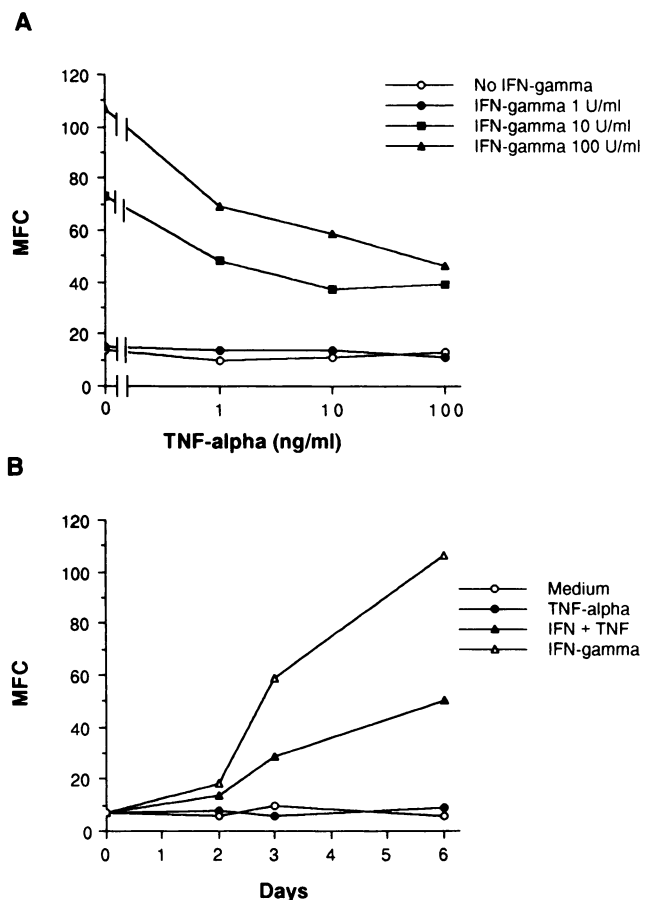

Figure 3. Dose response and time course of TNF-alpha inhibition of IFN-gamma-induced RA synoviocyte HLA-DR expression. (*A*) Synoviocytes were cultured for 72 h in various concentrations of IFN-gamma and TNF-alpha and analyzed for HLA-DR expression by FACS. (*B*) Synoviocytes were incubated for various times in medium, IFN-gamma (100 U/ml) and/or TNF-alpha (50 ng/ml). One representative experiment is shown in each panel ($n = 2$).

Role of prostaglandins in TNF-alpha inhibition of IFN-gamma effect. TNF-alpha induces PGE$_2$ production by synoviocytes (19) and this eicosanoid is known to inhibit HLA-DR expression on various cell types (29). For that reason, we studied a possible role of prostaglandins in TNF-alpha inhibition of IFN-gamma-mediated HLA-DR expression. Synoviocytes were incubated with both cytokines in the presence of 10^{-5} M indomethacin. No alteration in the inhibitory effect of TNF-alpha was observed (Fig. 4), suggesting that de novo prostaglandin synthesis is not involved. The fact that IL-1beta (which also increases prostaglandin synthesis by synoviocytes) did not affect IFN-gamma action also argues against a role for prostaglandins.

Sequential addition of IFN-gamma and TNF-alpha. Sequential addition experiments demonstrated that the inhibitory effect of TNF-alpha on HLA-DR induction only occurred when synoviocytes were exposed to IFN-gamma and TNF-alpha simultaneously. Fig. 5 *A* shows a representative experiment ($n = 3$) in which synoviocytes preincubated for 12 h with TNF-alpha, washed, and then recultured in IFN-gamma alone had normal HLA-DR induction. Similarly, when synoviocytes were preincubated with IFN-gamma for 12 h, washed, and
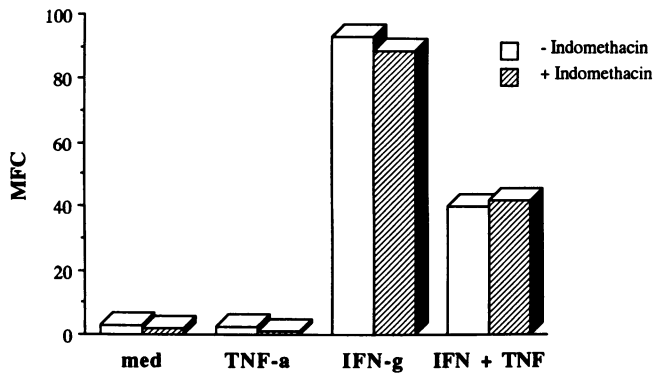
Figure 4. Effect of indomethacin on TNF-alpha inhibition of IFN-gamma-mediated synoviocyte HLA-DR expression. RA synoviocytes were cultured for 72 h in medium, IFN-gamma (100 U/ml), TNF-alpha (50 ng/ml), or both. Cultures were incubated in the presence or absence of indomethacin (10^{-5} M). One representative experiment is shown ($n = 3$).

then treated with TNF-alpha, HLA-DR induction was not inhibited (data not shown). Addition of TNF-alpha at various time points during continuous IFN-gamma stimulation was inhibitory only in the first 24 h of IFN-gamma treatment ($n = 3$) (Fig. 5 B).
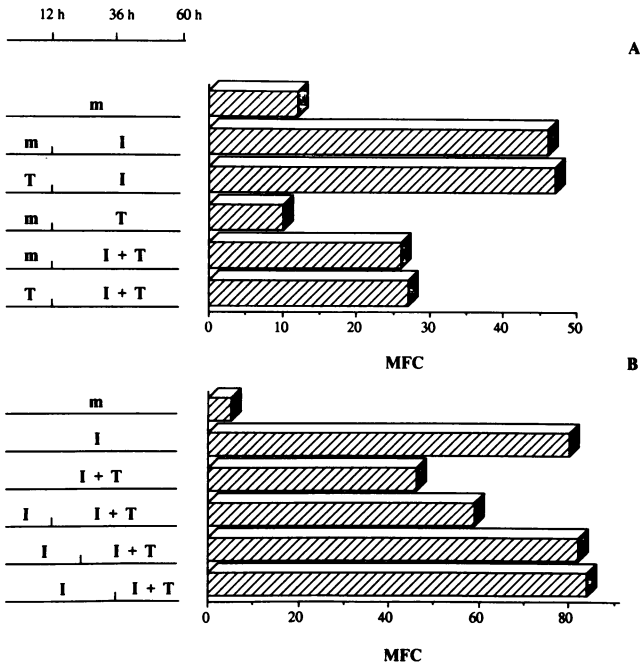

Figure 5. Effect of the sequential addition of IFN-gamma (100 U/ml) (*I*) and TNF-alpha (50 ng/ml) (*T*) on synoviocyte HLA-DR expression. (A) Preincubation with TNF-alpha. Synoviocytes were cultured for 12 h in the presence of medium (*m*) or TNF-alpha and then washed. The synoviocytes were cultured for an additional 48 h with IFN-gamma and/or TNF-alpha. Cells were then analyzed for HLA-DR expression by FACS. (B) Addition of TNF-alpha at various time points after IFN-gamma. Synoviocytes were cultured for 60 h with medium alone or with 100 U/ml of IFN-gamma. TNF-alpha was added at various times without removing the IFN-gamma. HLA-DR was then measured by FACS.
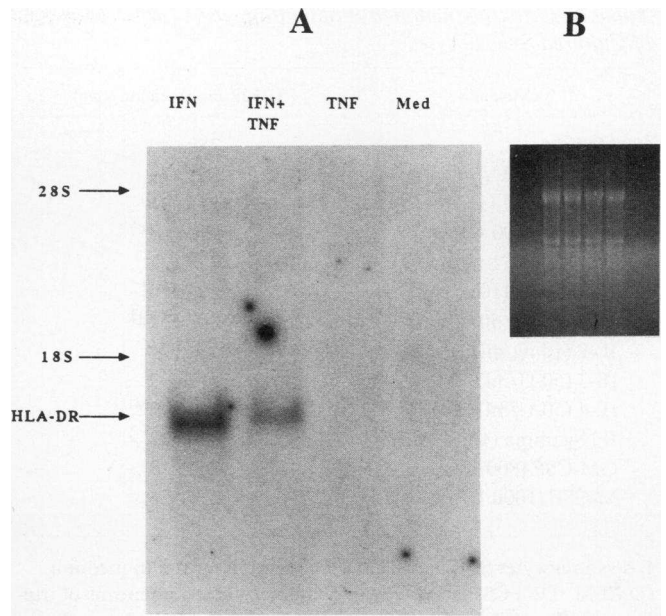

Figure 6. Inhibitory effect of TNF-alpha on IFN-gamma-induced synoviocyte HLA-DR beta chain mRNA accumulation. (A) RA synoviocytes were cultured for 72 h in medium, IFN-gamma (100 U/ml), TNF-alpha (50 ng/ml), or both. Total cytoplasmic RNA was isolated and assayed by Northern blot analysis. 20 μg of RNA was loaded in each lane. (B) Ethidium bromide staining of ribosomal RNA was used as control for the amount of RNA loaded.

Effect of TNF-alpha on HLA-DR mRNA accumulation. Northern blot analysis was performed to study the mechanism of TNF-alpha inhibition of HLA-DR induction. Cytoplasmic RNA prepared from synoviocytes cultured in medium, IFN-gamma, TNF-alpha, or IFN-gamma plus TNF-alpha for 2 d was assayed for HLA-DR mRNA. Fig. 6 shows that TNF-alpha inhibited HLA-DR mRNA accumulation in IFN-gamma-treated cells, indicating that the effect is likely pretranslational.

IFN-gamma/TNF-alpha combination is not cytotoxic. Cytotoxicity induced by a combination of TNF-alpha and IFN-gamma has been reported in human umbilical vein endothelial cells (30). To address the possibility that a similar mechanism could be involved in the inhibitory effect of TNF-alpha on IFN-gamma function, we studied the influence of TNF-

Table I. TNF-alpha/IFN-gamma Is Not Cytotoxic

		Cells × 10^{-5}	% viable
ST1	Medium	1.25	98.7
	TNF/IFN	1.20	98.8
ST2	Medium	1.17	99.4
	TNF/IFN	1.18	99.1
ST3	Medium	1.15	98.6
	TNF/IFN	1.16	97.8

10^5 synoviocytes from three separate RA ST were cultured for 72 h in the presence of medium alone or TNF-alpha (100 ng/ml) plus IFN-gamma (100 U/ml). Cells were counted in a hemocytometer and viability assessed by trypan blue dye exclusion.

Table II. Cytokine-mediated Proliferation of Cultured Synoviocytes

Cytokine	[³H]TdR incorporation (cpm)
Medium	259±40
IL-1beta (1 U/ml)	289±32
IL-1beta (10 U/ml)	325±31
IL-1beta (100 U/ml)	558±18*
TNF-alpha (1 ng/ml)	300±22
TNF-alpha (10 ng/ml)	805±70‡
TNF-alpha (50 ng/ml)	1,208±214‡
IL-2 (10 ng/ml)	232±22
IL-3 (50 U/ml)	273±32
IL-4 (50 U/ml)	247±10
IFN-gamma (100 U/ml)	280±37
GM-CSF (800 U/ml)	242±19
M-CSF (1000 U/ml)	275±35

RA synoviocytes (third passage) were cultured for 4 d in medium (DMEM 10% FCS) alone or with cytokine. Values are means of triplicate cultures±SEM. One representative experiment is shown. Each cytokine was tested three to seven times in separate experiments. * $P < 0.05$, ‡ $P < 0.001$, compared to medium.

alpha/IFN-gamma on synoviocyte viability. As shown in Table I, the combination of the two cytokines was not toxic to synoviocytes after a 72-h exposure.

Proliferation of cultured synoviocytes

Effect of individual cytokines. Cytokines were tested for their ability to induce proliferation of cultured synoviocytes. Table II shows a representative experiment. TNF-alpha consistently increased tritiated thymidine uptake by cultured synoviocytes (stimulation index = 3.60±1.03, $n = 7$, $P < 0.001$). In four of seven cases, IL-1beta also stimulated synoviocyte DNA synthesis (stimulation index = 2.31±0.46, $P < 0.05$). However, little or no stimulation was observed in cells from three other RA tissues studied. In general, IFN-gamma had no effect on DNA synthesis (stimulation index = 1.12±0.14, $n = 7$, $P > 0.1$), although in two of seven samples there was a modest increase in tritiated thymidine uptake. IL-2 (1–100 ng/ml), IL-3 (1–100 U/ml), IL-4 (1–100 U/ml), GM-CSF (8–800 U/ml), and M-CSF (10–1,000 U/ml) did not alter synoviocyte tritiated thymidine incorporation. The results were similar with RA and OA synoviocytes (data not shown).

Cytokine interactions. The effect of different cytokine combinations on synoviocyte proliferation was evaluated. IL-1 and TNF-alpha had an additive effect (see Table III for a representative experiment). The most novel finding was the marked inhibition of TNF-alpha-induced proliferation by IFN-gamma (70±14% inhibition, $n = 6$, $P = 0.002$) (see Table IV, experiment 1, for a representative experiment). The inhibitory effect of IFN-gamma on TNF-alpha was also observed when cells were starved in 1% FCS for 3 d before the addition of cytokines (see Table IV, experiment 2, for a representative experiment). It is interesting that IFN-gamma did not inhibit the proliferative action of IL-1 (see Table IV). A representative dose response of IFN-gamma-mediated inhibition of synoviocyte proliferation is shown in Table IV, experiment 3. IL-2 (100 ng/ml), IL-3 (100 U/ml), IL-4 (100 U/ml), GM-CSF (800

Table III. TNF-alpha and IL-1beta Interaction on Cultured Synoviocyte Proliferation

Cytokine	[³H]TdR incorporation (cpm)
Medium	372±45
IL-1beta	1,087±117
TNF-alpha	1,218±79
IL-1beta + TNF-alpha	2,207±34*

RA synoviocytes (fifth passage) were cultured for 4 d in medium (DMEM 10% FCS) alone or with cytokine (IL-1 beta 50 U/ml; TNF-alpha 50 ng/ml). Values are means of triplicate cultures±SEM. One representative experiment ($n = 3$) is shown. * $P < 0.001$ compared to TNF-alpha.

U/ml), and M-CSF (1,000 U/ml) had no effect on the proliferative activity of IL-1 or TNF-alpha (data not shown).

Role of prostaglandins in IFN-gamma inhibition of TNF-alpha effect. PGE_2 can inhibit cellular proliferation (31) and culturing the cells in the presence of indomethacin enhances the proliferative effects of TNF-alpha and IL-1 (14, 17), perhaps reflecting the capacity of these two cytokines to induce PGE_2 production (17–19). To evaluate a possible role of prostaglandins in the inhibitory effect of IFN-gamma, synoviocytes were cultured in the presence of cytokines plus or minus indomethacin. As expected, indomethacin (10^{-5} M) increased the proliferation of TNF-alpha-stimulated synoviocytes; however, it did not modulate IFN-gamma inhibition of the TNF-alpha effect (see Table V for a representative experiment).

Table IV. Inhibition of TNF-alpha-induced Synoviocyte Proliferation by IFN-gamma

Cytokine	[³H]TdR incorporation (cpm)	
	Exp. 1	*Exp. 2*
Medium	259±40	770±110
IFN-gamma	281±33	990±240
TNF-alpha	1,208±214	1,480±70
TNF-alpha + IFN-gamma	279±95*	800±202‡
IL-1beta	558±18	ND
IL-1beta + IFN-gamma	510±35	ND
		Exp. 3
Medium		1,126±71
TNF-alpha§		2,062±40
TNF-alpha + IFN-gamma (1 U/ml)		1,845±159
TNF-alpha + IFN-gamma (10 U/ml)		1,233±186‡
TNF-alpha + IFN-gamma (100 U/ml)		945±130*
IFN-gamma (100 U/ml)		980±50

Exp. 1 and 2. Synoviocytes were cultured for 4 d in medium alone or with cytokine (IL-1beta 50 U/ml; TNF-alpha 50 ng/ml; IFN-gamma 100 U/ml). Experiment 1 was performed in DMEM-10% FCS and experiment 2 was carried out in DMEM-1% FCS. Representative experiments are shown ($n = 3$ in 10% FCS and $n = 3$ in 1% FCS). Exp. 3. Dose response in DMEM-10% FCS. § TNF-alpha 50 ng/ml. Values are means of triplicate cultures±SEM. * $P < 0.001$; ‡ $P < 0.01$ compared with TNF-alpha.

Table V. Effect of Indomethacin on Cytokine-induced Synoviocyte Proliferation

	[³H]TdR incorporation (cpm)	
Cytokine	−Indomethacin	+Indomethacin
Medium	490±91	538±59
TNF-alpha	1,954±256	2,670±240
IFN-gamma	570±143	585±210
TNF-alpha + IFN-gamma	1,130±210	1,010±320

Synoviocytes (third passage) were cultured for 4 d in medium (DMEM 10% FCS) alone or cytokines (IFN-gamma 100 U/ml; TNF-alpha 50 ng/ml) in the presence or absence of indomethacin 10^{-5} M. Values are means of triplicate cultures±SEM. One representative experiment is shown ($n = 4$).

Table VI. Cytokine Interactions on Synoviocyte Collagenase Production

Cytokine	Collagenase (U/ml)
Medium	≤0.10
IL-1beta	0.99±0.01
TNF-alpha	0.36±0.03
IL-1beta + TNF-alpha	1.11±0.04
TNF-alpha + IFN-gamma	≤0.10*
IL-1beta + IFN-gamma	1.09±0.09

RA synoviocytes (fourth passage) were cultured for 3 d in DMEM-1% FCS and cytokines (IL-1 50 U/ml; TNF-alpha 50 ng/ml; IFN-gamma 100 U/ml). The supernatants were harvested and assayed for collagenase by indirect ELISA. Values are means of triplicate wells±SEM. One representative experiment is shown ($n = 3$).
* $P < 0.01$ compared with TNF-alpha.

Collagenase activity in supernatants of cultured synoviocytes

Effect of individual cytokines. Collagenase activity in supernatants of synoviocytes cultured in the presence of different cytokines was measured by an indirect ELISA. Our data (see Fig. 7 for a representative experiment) confirm that IL-1 and TNF-alpha increase synoviocyte production of collagenase (from < 0.10 U/ml to 1.10±0.15, $n = 5$, $P = 0.003$, and 0.72±0.24, $n = 6$, $P = 0.048$, respectively) and demonstrate the narrow spectrum of cytokines with this capacity. IL-2, 1–100 ng/ml; IL-3, 1–100 U/ml; IL-4, 1–100 U/ml; IFN-gamma, 1–100 U/ml; GM-CSF, 8–800 U/ml; and M-CSF, 10–1,000 U/ml, did not enhance collagenase activity.

Cytokine interactions. To study the possibility that other cytokines might interfere with the increase in collagenase activity in the supernatants of IL-1 and TNF-alpha-treated synoviocytes, we analyzed the effect of several combinations. Table VI shows that IL-1beta and TNF-alpha together had a less than additive effect. It is interesting that IFN-gamma decreased TNF-alpha-mediated collagenase activity (69±19% inhibition, $n = 3$, $P = 0.029$) but had no effect on the action of IL-1 (see Table VI for a representative experiment). Studies are currently in progress to determine if the decrease in collage-
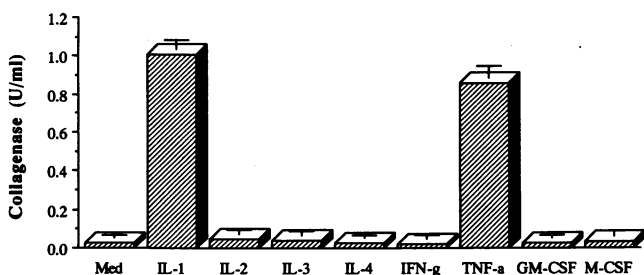
nase activity is due to diminished collagenase production or increased tissue inhibitor of metalloproteinases production. IL-2, IL-3, IL-4, GM-CSF, and M-CSF had no effect on TNF-alpha or IL-1 mediated-collagenase production (data not shown).

GM-CSF production by cultured synoviocytes

We have recently observed that TNF-alpha (1–100 ng/ml) induces cultured synoviocytes to express the GM-CSF gene and secrete immunoreactive protein (Alvaro-Gracia, J. M., N. J. Zvaifler, and G. S. Firestein, submitted for publication). To test whether IFN-gamma inhibits GM-CSF production, RA synoviocytes (passage 3–7) from four separate donors were incubated for 48 h in the presence of medium, TNF-alpha, IFN-gamma, TNF-alpha plus IFN-gamma, IL-1beta, or IL-1beta plus IFN-gamma. GM-CSF was assayed in the supernatants by solid phase ELISA. TNF-alpha and IL-1beta alone induced GM-CSF production; IFN-gamma blocked only the effect of TNF-alpha (see Table VII).

Discussion

Most studies on the effects of cytokines on synoviocyte function have been concerned primarily with defining the activities of individual factors (13–21). However, it is now clear that these mediators function in concert in the synovium and that

Figure 7. Collagenase activity in supernatants of cytokine stimulated RA synoviocytes. Synoviocytes (third passage) were cultured for 72 h in DMEM-1% FCS and the following cytokines: IL-1beta (100 U/ml), IL-2 (50 ng/ml), IL-3 (100 U/ml), IL-4 (100 U/ml), IFN-gamma (100 U/ml), TNF-alpha (50 ng/ml), GM-CSF (800 U/ml), and M-CSF (1,000 U/ml). The supernatants were harvested and assayed for collagenase activity by an indirect ELISA. Results are indicated as units/ml per 10^6 cells. One representative experiment is shown ($n = 3$ to 7 for each cytokine).

Table VII. IFN-gamma Inhibition of TNF-alpha-induced GM-CSF Production by Cultured Synoviocytes

	ST4	ST5	ST6	ST7
Medium	<5*	<5	<5	<5
TNF-alpha (100 ng/ml)	260	122	164	1264
IFN-gamma (100 U/ml)	<5	<5	<5	<5
TNF-alpha + IFN-gamma	<5	<5	<5	346
IL-1beta (100 U/ml)	210	118	ND	ND
IL-1beta + IFN-gamma	290	164	ND	ND

2×10^5 synoviocytes from four separate synovial tissues were cultured in 0.5 ml medium with or without cytokine for 48 h. Supernatants were harvested and assayed by ELISA.
* pg/ml of GM-CSF.

specific effects of a single cytokine depend on the presence (or absence) of other factors. For this reason, the major goal of this study was to identify and characterize cytokine interactions that might play a role in synovitis. The additive effects observed between IL-1 and TNF-alpha in our study were not surprising; these two proteins frequently enhance each other's function and even show synergy in fibroblast PGE$_2$ production (32) or induction of rabbit experimental arthritis (33). However, the finding of mutual antagonism between TNF-alpha and IFN-gamma in four separate systems is novel and provocative. This phenomenon appears to be specific for TNF-alpha and IFN-gamma, since similar interactions were not observed between IL-1 and IFN-gamma in our study or in a recent study of proliferation and prostaglandin production by RA synoviocytes (34). The effect of other cytokines, including M-CSF, GM-CSF, IL-2, IL-3, and IL-4, on the stimulatory actions of IL-1beta, TNF-alpha, and IFN-gamma were also studied. None of them influenced synoviocyte HLA-DR expression, proliferation, or collagenase production. The lack of inhibition of IL-1 on HLA-DR expression differs from a previous study on rat synoviocytes in which IL-1 (but not TNF-alpha) blocked HLA-DR induction by IFN-gamma (35). The reason for the discrepancy is not known, but might reflect species differences.

The interactions between IFN-gamma and TNF-alpha on synoviocytes are striking, particularly in light of previous studies on other cell types in which IFN-gamma and TNF-alpha were either synergistic or additive. Examples of this phenomenon include the induction of HLA-DR molecules on a murine macrophage cell line (36) and various human tumor cell lines (37–39); induction of chemotactic peptide receptor expression on HL-60 cells (a human promyelocytic cell line) (40); induction of class I MHC molecules on human glioma tumor cells (41); induction of glycosaminoglycan synthesis by human lung fibroblasts (42); and increased production of collagen by dermal fibroblasts (43). TNF-alpha inhibits IFN-gamma-mediated induction of class II MHC molecules on human umbilical vein endothelial cells (44), but the fact that this combination is also cytotoxic on those cells (30) makes the interpretation of those data difficult. In contrast, we demonstrated that TNF-alpha and IFN-gamma inhibit each other's effect on cultured synoviocytes without cytotoxicity despite the fact that they both have stimulating effects when used independently. For instance, TNF-alpha significantly inhibits IFN-gamma-mediated HLA-DR induction, while IFN-gamma blocks TNF-alpha-mediated synoviocyte proliferation, collagenase production, and GM-CSF production. The effect is not unique to cells isolated from RA synovium since OA synoviocytes behaved in a similar fashion.

The mechanism of TNF-alpha antagonism of IFN-gamma-mediated HLA-DR induction is not known. Studies of HLA-DR mRNA expression in synoviocytes indicate that the inhibition is pretranslational, although it is not known whether changes in gene transcription or mRNA degradation are responsible. Prostaglandin synthesis was considered as a possible explanation (particularly because TNF-alpha is known to induce PGE$_2$ production by synoviocytes [19]), but this is an unlikely explanation since indomethacin does not interfere with the inhibitory effects. Another potential inhibitory mechanism could be the modulation of the surface IFN-gamma receptor expression; however, preincubation of synoviocytes with TNF-alpha did not inhibit HLA-DR induction, suggest-

ing that this is not the mechanism responsible for the inhibitory effect of TNF-alpha. Furthermore, in preliminary experiments with ^{125}I-IFN-gamma, TNF-alpha does not downregulate specific IFN-gamma binding to cultured synoviocytes (unpublished data).

The data on HLA-DR expression on synoviocytes might provide some explanations for the distribution of this class II MHC antigen in the rheumatoid synovium. Although there is some disagreement, most evidence suggests that type B synoviocytes express little HLA-DR (considerably less than synovial macrophages and type A synoviocytes) (22, 45–47). We have recently shown that GM-CSF, which is present in the rheumatoid joint (28), plays a major role in macrophage HLA-DR and DQ expression in RA, possibly in combination with TNF-alpha (20). However, of the cytokines tested, only IFN-gamma induces HLA-DR on the fibroblast-like synoviocytes. Since only very small amounts of IFN-gamma are produced in the synovium (23, 48), it is not surprising, therefore, to find low surface HLA-DR expression on fibroblast-like synoviocytes. Furthermore, the small amount of IFN-gamma that is present is likely antagonized by TNF-alpha (10), hence contributing to the relative lack of class II MHC molecules on these cells.

The mechanism of IFN-gamma inhibition of TNF-alpha effects is also unknown. Although prostaglandins are known to inhibit synoviocyte proliferation (14, 17), experiments with indomethacin suggest that they are not involved. There is no information on TNF-alpha receptor modulation on synoviocytes, although in most cells studied IFN-gamma actually increases specific TNF-alpha surface binding (49–51). It seems unlikely that the inhibitory effect on TNF-alpha-induced proliferation reflects a generalized antiproliferative action by IFN-gamma (52, 53) since this cytokine alone did not inhibit synoviocyte proliferation. In fact, it modestly stimulated synoviocyte proliferation in some experiments, a phenomenon that has been described by others (14, 54), and did not interfere with IL-1-mediated synoviocyte proliferation. It is also unlikely that cytotoxicity or interactions with factors present in fetal calf serum account for the inhibitory effect (IFN-gamma also inhibited TNF-alpha-mediated proliferation of serum starved synoviocytes).

The observed in vitro antagonism between IFN-gamma and TNF-alpha suggests a novel homeostatic control mechanism. Since each cytokine interferes with the synoviocyte-stimulating effects of the other, their combined presence might be one method by which articular inflammation is normally attenuated. This could be particularly pertinent in RA where T cells have a specific defect in IFN-gamma production (55). The RA synovium produces very little IFN-gamma and only very low levels are found in synovial effusions (23, 48). In contrast, large amounts of TNF-alpha can be detected in synovial effusions (10) and over 5% of ST cells express the TNF-alpha gene (48). The absence of IFN-gamma under these circumstances could result in unopposed stimulation of fibroblast-like type B synoviocytes by TNF-alpha, leading to local proliferation, pannus formation, as well as GM-CSF and collagenase production. GM-CSF has been implicated in the activation of synovial macrophages (20) while collagenase is involved in matrix degradation in the joint. This hypothesis provides one possible explanation for the reported (albeit modest) efficacy of IFN-gamma in RA therapy (56, 57). Perhaps the benefits are limited because IFN-gamma, like GM-CSF, can induce class II

MHC antigens on macrophages and does not antagonize the effects of IL-1, which is also present in high concentrations in the inflamed joint. A better understanding of these mechanisms might provide a rational basis for studying specific combinations of cytokines and inhibitors in the therapy of RA and other forms of chronic inflammatory arthritis.

Acknowledgments

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