Effects of interferon gamma on cultured synovial cells from patients with rheumatoid arthritis: inhibition of cell growth, prostaglandin E₂, and collagenase release*

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Abstract

The effects of recombinant interferon gamma (rIFN γ) on the in vitro growth of adherent synovial fibroblast-like cells from patients with rheumatoid arthritis (RA) and also on the release of prostaglandin E₂ and collagenase from these cells stimulated with recombinant interleukin-1 β (rIL-1 β) were investigated. The growth of adherent synovial cells from six of nine samples, determined by [³H]thymidine incorporation, was inhibited by rIFN γ in a manner dependent on dose. The release of prostaglandin E₂ and collagenase from adherent synovial cells stimulated with rIL-1 β was also suppressed by rIFN γ in all samples tested, though the basal release of these inflammatory mediators was little influenced. No apparent correlation between inhibition of proliferation by rIFN γ and either inhibition by rIFN γ of rIL-1 β stimulated prostaglandin E₂ release or the endogenous synthesis of prostaglandins was found.

Interferon gamma (IFN γ) has been shown to have antiproliferative, immunomodulatory, and antiviral properties.¹⁻³ The ability of IFN γ to induce and enhance the expression of class II major histocompatibility complex antigens on various types of cells is generally believed to be a factor in the pathogenesis of autoimmune disorders such as rheumatoid arthritis (RA).^{3,4} Recent clinical investigations have shown some efficacy of recombinant IFN γ (rIFN γ) in the treatment of RA.⁵⁻⁷ Moreover, a number of reports have shown diminished production of IFN γ by peripheral blood or synovial fluid lymphocytes from patients with RA,⁸⁻¹³ and the presence of only low concentrations of IFN γ in rheumatoid synovial fluids and tissues,¹³⁻¹⁵ though contradictory results have also been reported.^{16,17}

One of the features of RA is the hyperplasia of synovial lining cells and fibroblasts associated with the infiltration of lymphocytes and monocytes/macrophages into affected joints. Inflamed tissues of RA synovium release potent interleukin-1 (IL-1) activity,¹⁸⁻²⁰ which in turn activates the synovial cells to generate large amounts of tissue degrading mediators, such as collagenase and prostaglandins.^{21,22} The role of IFN γ in relation to these aspects of RA, however, has not been fully investigated.

This study was designed to investigate the influence of rIFN γ on the in vitro proliferation of adherent synovial fibroblast-like cells from patients with RA and the release of prosta-

glandins and collagenase from these cells stimulated with recombinant interleukin-1 β (rIL-1 β). The results show the inhibitory effects of rIFN γ on both cell proliferation and the rIL-1 β induced release of prostaglandin and collagenase activity. These results suggest a further aspect of the regulatory role of IFN γ in chronic inflammatory disease.

Materials and methods

SOURCE AND PREPARATION OF ADHERENT SYNOVIAL CELLS

Specimens of synovium were obtained at the time of synovectomy from the knee or hip joint of nine patients with classical or definite RA according to the American Rheumatism Association criteria.²³ Patients who were taking more than 3 mg/day of prednisolone or 300 mg/day of D-penicillamine were excluded from the study. Adherent synovial cells were prepared from the synovium specimens essentially as has been described previously.^{24,25} Briefly, synovium was minced and dissociated enzymatically into a single cell suspension by treatment with bacterial collagenase (type I; Sigma Chemical Co, St Louis, MO, USA). The cells suspended in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco) and antibiotics (abbreviated as DMEM-FCS) were filtered through sterile gauze and plated at ca 10⁵/cm² as primary cultures into 75 cm² tissue culture flasks (Falcon 3024, Becton Dickinson and Co, Lincoln Park, NJ, USA). Adherent synovial cells were derived by trypsinisation (0.25% trypsin/0.02% EDTA) and passage (1:3 to 1:4) in DMEM-FCS of the primary cultures. Most adherent synovial cells were morphologically fibroblast-like, in agreement with previous reports.^{25,26} The cells were used usually between the second and fourth passages.

rIFN γ , rIL-1 β , AND ANTISERUM TO rIFN γ

Recombinant interferon gamma (3.1 $\times$ 10⁷ anti-viral U/mg protein) from transfected *E coli* and rabbit immune serum against rIFN γ were provided by Genentech (South San Francisco, CA, USA). Recombinant interleukin-1 β (10⁸ U/mg) was purchased from Genzyme (Boston, MA, USA).

[³H]THYMIDINE INCORPORATION ASSAY

Adherent synovial cells obtained from the con-

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fluent cultures were plated into 96-well flat bottomed plates (Falcon 3072, Becton Dickinson and Co) at 5×10^3 cells/well in 0.1 ml of DMEM-FCS. After two days of incubation at 37°C in a 5% CO₂ atmosphere the medium was removed and replaced with 0.1 ml of fresh DMEM-FCS containing rIFN γ or other agents, in triplicate, and incubated for an additional three days. Cultures were pulsed with 18.5 kBq [³H]thymidine (New England Nuclear, Boston, MA, USA) for the last 18 hours of incubation, and the incorporation of [³H]thymidine into DNA was assessed.^{27, 28} Briefly, after medium aspiration cultures were trypsinised, harvested onto glass fibre filters with an automated harvester, and lysed by washing with distilled water. The DNA labelled with [³H]thymidine, trapped by the glass fibre filters, was counted by liquid scintillation spectrometry.

PRELABELLED [³H]THYMIDINE RELEASE ASSAY
The cytolytic potential of rIFN γ was assessed by a slight modification of a method previously reported.²⁹ Briefly, adherent synovial cells in the exponential growth phase were incubated in DMEM-FCS supplemented with 37 kBq/ml of [³H]thymidine for two days at 37°C in a 5% CO₂ atmosphere. After trypsinisation 5×10^3 [³H]-thymidine labelled cells suspended in DMEM-FCS were distributed into 96-well plates and incubated with rIFN γ for one to three days. The cells were harvested onto glass fibre filters and the radioactivity remaining in the cells was counted as described above.

DETERMINATION OF PROSTAGLANDIN E₂, 6-KETOPROSTAGLANDIN F_{1 α} , AND THROMBOXANE B₂
To measure prostanoid release rheumatoid adherent synovial cells were cultured as described previously.²⁶ Briefly, 5×10^4 adherent synovial cells in 0.3 ml of DMEM-FCS were placed in 24-well trays (Falcon 3047, Becton Dickinson and Co) in duplicate. After two days of incubation at 37°C in a 5% CO₂ incubator the medium was removed and replaced with the same volume of fresh DMEM-FCS containing rIFN γ either alone or in combination with rIL-1 β , and incubated for an additional three days. Culture supernatants were harvested, pooled, and analysed for concentrations of prostaglandin E₂, 6-ketoprostaglandin F_{1 α} , and thromboxane B₂ by radioimmunoassay kits (New England Nuclear). Assays were performed in duplicate according to the manufacturer's instructions.

COLLAGENASE ASSAY
Three-day culture supernatants of adherent synovial cells, obtained as described above, were treated with 0.2 mg/ml of TPCK-trypsin (Sigma Chemical Co) at 25°C for 10 minutes, followed by soybean trypsin inhibitor (Sigma Chemical Co) and incubation for 10 minutes to convert the collagenase into its active form.²⁴ Collagenase activity was measured in duplicate³⁰ with fluorescein isothiocyanate labelled collagen (Cosmo Bio Co, Tokyo, Japan) as a substrate.

As fluorescein isothiocyanate is not released by trypsin in this assay any release of the isothiocyanate reflects actual collagenase activity. One unit of collagenase activity was defined as the amount of enzyme degrading 1 μ g of collagen/min under the conditions used.³⁰

Results
EFFECT OF rIFN γ ON THE PROLIFERATION OF CULTURED ADHERENT SYNOVIAL CELLS FROM PATIENTS WITH RA
Isolated synovial fibroblast-like cells, obtained from nine different patients with RA, were cultured with various concentrations of rIFN γ for three days, and [³H]thymidine incorporation for the last 18 hours of incubation was determined. Figure 1 shows that [³H]thymidine incorporation in three-day cultures of six (patients 1, 2, 3, 4, 6, and 9) of nine samples was inhibited by rIFN γ in a manner dependent on dose. The other three samples (patients 5, 7, and 8) showed only marginal response to rIFN γ in three-day cultures. The data in fig 2 show

Figure 1: Effect of recombinant interferon gamma (rIFN γ) on [³H]thymidine ([³H]TdR) incorporation by adherent synovial cells from patients with rheumatoid arthritis. Triplicate cultures of adherent synovial cells were treated with rIFN γ at concentrations of 1 to 10⁴ U/ml for three days, and were pulsed with [³H]TdR for the last 18 hours of incubation. Patient number is indicated at the right.

Co-additive	rIFN γ (U/ml)	[³ H] TdR incorporation (dpm/well $\times 10^{-3}$)			
		0	2	4	6
-	0	~55			
	100	~35			
Normal serum	0	~55			
	100	~35			
Anti-rIFN γ serum	0	~55			
	100	~55			

Figure 2: Effect of antibody to recombinant interferon gamma (anti-rIFN γ) on the antiproliferative effect of rIFN γ . Triplicate cultures of adherent synovial cells from patient No 1 with rheumatoid arthritis were treated for three days with 100 U/ml of rIFN γ in combination with either anti-rIFN γ rabbit serum at 1/200 dilution or normal rabbit serum (1/200). Mean and SD are shown. [³H]TdR=[³H]thymidine.

that the antiproliferative effect of rIFN γ was abrogated completely by the coaddition of antibody to rIFN γ , confirming that IFN γ is responsible for the inhibition of rheumatoid synovial cell proliferation. No direct cytolytic activity of rIFN γ against synovial cells could be shown by using the prelabelled [3 H]thymidine release assay as well as the trypan blue exclusion test (data not shown).

The following experiments were undertaken to examine the participation of endogenous prostaglandins in the antiproliferative effect of rIFN γ . As shown representatively in fig 3, neither the addition of 5 μ g/ml indomethacin, a cyclo-oxygenase inhibitor, nor 0.03 ng/ml of rIL-1 β to the culture influenced the antiproliferative effect of rIFN γ against rheumatoid adherent synovial cells, though the basal rate of proliferation was sometimes modified, albeit marginally. As the amounts of prostaglandin released from the cells were drastically changed by the drugs (see below) these results indicate that inhibition of proliferation by rIFN γ is not related to prostaglandin synthesis.

EFFECT OF rIFN γ ON PROSTAGLANDIN E $_2$ RELEASE FROM CULTURED ADHERENT SYNOVIAL CELLS FROM PATIENTS WITH RA

The synovial fibroblast-like cells from patients with RA were cultured with rIFN γ or rIL-1 β , or both, for three days, and prostaglandin E $_2$ concentration in the supernatant fluid was assayed. The solid lines in fig 4 show that basal concentrations of prostaglandin E $_2$ released spontaneously into the culture supernatants were very low and were little influenced by rIFN γ . The release of prostaglandin E $_2$ was increased 10 to 500-fold, however, by stimulation with rIL-1 β (0.03–0.1 ng/ml), and this stimulated release was reduced by between 45 and 85% by the coaddition of rIFN γ in all samples tested, as shown by the broken lines in fig 4. The extent of inhibition of rIL-1 β stimulated prostaglandin E $_2$ release by rIFN γ was compared with that of the inhibition of proliferation by rIFN γ in each sample of adherent synovial cells, but no correlation was found.

Table 1 shows that rIFN γ inhibited the rIL-1 β induced release of the other prostanoids measured—namely, 6-ketoprostaglandin F $_{1\alpha}$ and thromboxane B $_2$, in a manner quantitatively

similar to the inhibition of prostaglandin E $_2$ release.

EFFECT OF rIFN γ ON COLLAGENASE RELEASE FROM CULTURED ADHERENT SYNOVIAL CELLS FROM PATIENTS WITH RA

Collagenase activity, released in a latent form in the culture supernatants of adherent synovial cells, was determined after activation with trypsin. Spontaneous release of collagenase activity was almost negligible in all the cultures tested. In cultures of most cell samples, 0.03 ng/ml of rIL-1 β , which was adequate for stimulation of prostaglandin E $_2$ release, was insufficient to induce the release of collagenase

Figure 4: Effect of recombinant interferon gamma (rIFN γ) on prostaglandin E $_2$ (PGE $_2$) release from adherent synovial cells from patients with rheumatoid arthritis. Duplicate cultures of adherent synovial cells were treated with rIFN γ at concentrations of 1 to 10 4 U/ml either alone (solid lines) or in combination with recombinant interleukin-1 β (rIL-1 β) (broken lines; 0.03 ng/ml of rIL-1 β for samples from patients 2, 3, 4, 7, 8, and 9, and 0.1 ng/ml of rIL-1 β for samples from patients 5 and 6). After three days of incubation the culture supernatants were harvested and PGE $_2$ concentrations were determined as described in 'Materials and methods'. Mean of duplicate determinations is plotted. Agreement between duplicates was $\pm$ 15% of the mean. Numerals at the right are the patient numbers.

Figure 3: Effect of indomethacin and recombinant interleukin-1 β (rIL-1 β) on the antiproliferative effect of recombinant interferon gamma (rIFN γ). Triplicate cultures of adherent synovial cells from patient No 2 with rheumatoid arthritis were treated for three days with 1 or 100 U/ml of rIFN γ in combination with either 5 μ g/ml of indomethacin or 0.03 ng/ml rIL-1 β . Mean and SD are shown. [3 H]TdR = [3 H]thymidine.

Table 1: Effect of recombinant interferon gamma (rIFN γ) on the recombinant interleukin-1 β (rIL-1 β) induced release of prostaglandin E $_2$ (PGE $_2$), 6-ketoprostaglandin F $_{1\alpha}$ (6-keto PGF $_{1\alpha}$), and thromboxane B $_2$ (TxB $_2$) from rheumatoid adherent synovial cells*

rIL-1 β (ng/ml)	rIFN γ (U/ml)	Prostanoids released (ng/ml)			
		PGE $_2$	6-keto PGF $_{1\alpha}$	TxB $_2$	
0	0	0.2†	0.4	1.7	
0.03	0	8.2	7.6	4.2	
0.03	1	4.9	4.3	1.6	
0.03	10	4.4	3.7	2.5	

*Adherent synovial cells from patient No 4 with rheumatoid arthritis were cultured with rIFN γ or rIL-1 β , or both for three days, and prostanoid concentrations in the culture supernatants were determined as described in 'Materials and methods'. †Values are means of duplicate determinations. Agreement between duplicates was $\pm$ 15% of the mean.

Table 2: Effect of recombinant interferon gamma (rIFN γ) on the recombinant interleukin-1 β (rIL-1 β) stimulated release of collagenase from rheumatoid adherent synovial cells*

rIL-1 β (ng/ml)	rIFN γ (U/ml)	Collagenase activity (U/ml)		
		Patient 6	Patient 3	Patient 9
0.00	0	<0.01†	<0.01	0.02
0.00	10 000	<0.01	<0.01	<0.01
0.03	0	0.11	0.01	<0.01
0.03	100	0.03	<0.01	<0.01
0.03	10 000	<0.01	<0.01	<0.01
0.30	0	0.52	0.08	4.70
0.30	10 000	0.07	<0.01	1.57

*Adherent synovial cells from patients with rheumatoid arthritis were cultured with rIFN γ or rIL-1 β , or both for three days, and collagenase activities in the culture supernatants were determined as described in 'Materials and methods'.

†Values are means of duplicate determinations. Agreement between duplicates was $\pm 10\%$ of the mean.

activity except for one sample (patient 6) (table 2). On the other hand, 0.3 ng/ml of rIL-1 β clearly induced the release of collagenase activity in all instances. This rIL-1 β induced release of collagenase activity was effectively reduced by either 10^2 or 10^4 U/ml of rIFN γ (table 2).

Discussion

This study shows some of the regulatory effects of IFN γ on the growth and functions of adherent synovial fibroblast-like cells from patients with RA by using IFN γ derived from recombinant DNA: rIFN γ inhibited not only cell proliferation in most samples, irrespective of their level of prostaglandin synthesis, but also the rIL-1 β induced prostaglandin E $_2$ release from all these cell samples. In addition, it also reduced the rIL-1 β induced collagenase release from these synovial cells.

This is the first demonstration of the inhibitory effect of rIFN γ on the growth of rheumatoid synovial fibroblast-like cells, although there are a few reports which show the antiproliferative effects of rIFN γ against dermal fibroblasts established from skin explants,²⁸ and transformed fibroblastic cell lines.³¹ In contrast with these results, Brinckerhoff and Guyre reported that rIFN γ increased the proliferation of synovial fibroblasts in culture.²⁵ One possible explanation of the various results may be differences of cell properties and cell population in culture, which depend on cell-donor individuality, passage number, and so on. Even in our own preliminary tests, both WI-38, a well known fibroblastic cell line resistant to IFN γ ,³¹ and one out of every two synovial cell samples obtained from patients with non-inflammatory joint diseases, showed slight increases in proliferation in response to low doses of rIFN γ (unpublished observation). All the samples of adherent synovial fibroblast-like cells in this study were obtained from patients with classical or definite rheumatoid arthritis,²³ and six of nine were clearly sensitive to the antiproliferative effect of rIFN γ when used at the second to fourth passages. Thus we conclude that at least for rheumatoid synovial fibroblast-like cells rIFN γ acts as a decelerator of cell proliferation in most instances. Nevertheless, the extent of the inhibition of proliferation by rIFN γ was

variable from sample to sample in our study. For a more precise analysis of these variations and of the discrepancy between studies further experiments with isolated subpopulations of adherent synovial cells would be needed.

Blocking of rIL-1 β induced prostaglandin E $_2$ release by rIFN γ has been reported for human peripheral monocytes.³² Our study shows the inhibition by rIFN γ of rIL-1 β induced prostaglandin release from rheumatoid synovial fibroblast-like cells. On the other hand, Amento *et al* reported an example in which rIFN γ influenced neither the basal release nor the crude monokine preparation-stimulated release of prostaglandin E $_2$ from cultured synovial cells collected from a patient with RA.²⁶ The discrepancy between their results and ours might be attributable to differences between the stimuli used, because the regulatory effects of rIFN γ on prostanoid release from human monocytes or murine macrophages have been shown to depend largely on the nature of the stimulus.³²⁻³⁵ Alternatively, the discrepancy might again be explained by differences in the properties of synovial cells, perhaps due to differences in the inflammatory condition of cell donors, because the extent of the inhibition of rIL-1 β stimulated prostaglandin E $_2$ release by rIFN γ has been shown to correlate negatively with the erythrocyte sedimentation rate of cell donor patients (unpublished observation).

Interleukin-1 has been shown to activate phospholipase A $_2$, generally believed to be a rate limiting enzyme in the generation of prostanoids, in rat fibroblasts³⁶ and rabbit chondrocytes.³⁷ Furthermore, Boraschi *et al*, who used zymosan stimulated macrophages, suggested that the inhibitory effect of IFN γ on prostanoid release was at the level of phospholipase A $_2$.³³ In fact, our finding that rIFN γ inhibited the release of 6-ketoprostaglandin F $_{1\alpha}$ and thromboxane B as well as prostaglandin E $_2$ to a similar extent suggests that rIFN γ exerts its inhibitory action at or before cyclo-oxygenase on the eicosanoid metabolic pathway. Growth control by endogenously produced prostaglandin E $_2$ is well reported.³⁸ The inhibition of prostanoid release by IFN γ , however, does not seem to have any direct correlation with its antiproliferative effect in our system because (a) rIFN γ inhibited cell proliferation without influencing the basal rate of prostaglandin E $_2$ release, and (b) neither the addition of indomethacin, a potent cyclo-oxygenase inhibitor, nor rIL-1 β influenced the antiproliferative effect of rIFN γ . Although the mechanisms are not fully understood, it is considered that the antiproliferative effects of interferons are related to their ability to lengthen all phases of the cell cycle.¹

Another finding in this study is the inhibition by rIFN γ of rIL-1 β induced release of collagenase activity from rheumatoid adherent synovial cells, though detailed investigations have yet to be done because of the fairly high concentration of rIL-1 β required to induce collagenase release. The inhibitory effect of rIFN γ on fibroblast collagen synthesis has been reported by several workers.^{26 28 39} It is interesting to mention that the synthesis of both a protease and its substrate—namely, collage-

nase and collagen, are both controlled by the same lymphokine, IFN γ . Although the sites of regulation by IFN γ in collagenase release are unknown at present, it is suggested that IFN γ blocks the transcriptional step of collagen synthesis.³⁹

The most important implication of these results concerns the role of IFN γ in the pathogenesis of RA. Rheumatoid synovial membrane organises into a replicating mass of tissue, or pannus, which is activated by IL-1 to destroy cartilage and bone by releasing mediators which degrade tissue.^{21 22} Our results suggest that IFN γ might contribute to retarding or terminating the progression of these destructive processes in rheumatoid joints by inhibiting synovial cell proliferation and IL-1 induced release of prostaglandins and collagenase. Antagonism by IFN γ of IL-1 stimulated bone resorption,^{40 41} interleukin-4 induced⁴² or Epstein-Barr virus induced⁴³ B cell activation, as reviewed by Browning,⁴⁴ might also be indicative of a down-regulatory role for IFN γ . Although it is essential to consider the overall role of IFN γ , including effects such as immunostimulation by enhancement of class II antigen expression^{2 3} or possible IL-1 production,⁴⁵ our results seem to provide a new perspective of the regulatory role of IFN γ in RA.

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