Clin. exp. Immunol. (1988) **72,** 67–73

The detection and initial characterization of colony-stimulating factors in synovial fluid

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(Accepted for publication 17 November 1987)

SUMMARY

In this study which included 16 patients with inflammatory or non-inflammatory arthropathies, human granulocyte-macrophage colony-stimulating activity was detected in synovial fluid. This was attributable to the presence of colony-stimulating factor(s) (CSF), as a direct action on human bone marrow progenitor cells was demonstrated using clone transfer experiments. Samples of synovial fluid also stimulated the growth of murine macrophage colonies and induced differentiation in the murine myelomonocytic leukemia cell line, WEHI-3B(D^+), which are characteristic properties of human macrophage-CSF or granulocyte-CSF respectively. These findings and the results of preliminary fractionation procedures suggested that the colony-stimulating activity in synovial fluid was not explicable by the presence of any one of the well-characterized human CSF acting in isolation. This provides a new insight into the pathogenesis of inflammatory arthropathies and supports the hypothesis that CSF have important roles *in vivo* in addition to the regulation of haemopoiesis.

Keywords colony-stimulating factors arthritis synovial fluid

INTRODUCTION

The colony-stimulating factors (CSF) are a group of glycoproteins which share the common property of inducing the lineage-specific growth of differentiated haemopoietic colonies from single bone marrow progenitor cells *in vitro* (Metcalf, 1984). Five human granulocyte-macrophage CSF have been identified and cDNAs encoding them have been genetically cloned. These predominantly act on either multipotential (Multi-CSF, interleukin 3; Yang *et al.*, 1986), granulocyte-macrophage (GM-CSF; Wong *et al.*, 1985), granulocyte (G-CSF; Nagata *et al.*, 1986), macrophage (M-CSF, CSF-1, human urinary CSF; Kawasaki *et al.*, 1985) or eosinophil (Eo-CSF, interleukin 5; Azuma *et al.*, 1986) lineages. However, the potential importance of CSF to the understanding of inflammatory joint diseases lies not so much in their ability to stimulate the proliferation and differentiation of haemopoietic progenitor cells, but in their ability to stimulate the functional activity of mature cells of the appropriate lineage.

The actions of recombinant human GM-CSF on mature neutrophils *in vitro* include chemotaxis (Wang *et al.*, 1987), inhibition of migration (Gasson *et al.*, 1984), stimulation of phagocytosis and antibody-dependent cytotoxicity, as well as enhancement of degranulation and superoxide production in response to a chemotactic stimulus (Lopez *et al.*, 1986; Weisbart *et al.*, 1985). Similar functional effects have been described in macrophages treated with M-CSF, GM-CSF or Multi-CSF *in vitro* and these actions could serve to trap and activate these cells at sites of inflammation. Furthermore, intraperitoneal administration of GM-CSF or Multi-CSF to mice results in a marked increase in the number and phagocytic capacity of their peritoneal macrophages (Metcalf *et al.*, 1986; 1987).

Rheumatoid arthritis (RA) is characterized by bone and cartilage destruction associated with synovial infiltration by macrophages and activated T cells (Ziff, 1974; Burmester *et al.*, 1983; Kurosaka & Ziff, 1983), both of which are known cellular sources of CSF. Furthermore, macrophages and neutrophils, the latter predominating in synovial fluid during acute exacerbations, release a variety of enzymes and mediators which could contribute to the local tissue damage and systemic features so typical of this disease (Weissman *et al.*, 1982). Since the response of these cells to CSF *in vitro* results in similar effects, it was thought that CSF may be mediators of joint inflammation *in vivo*. This study documents the detection of colony-stimulating activity in synovial fluid from inflamed joints and its attribution to the presence of colony-stimulating factors.

MATERIALS AND METHODS

Subjects

Synovial fluid and serum samples were obtained from patients where joint aspiration was diagnostically or therapeutically

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Table 1. Clinical data on the patients included in this study

Code	Sex	Age	Diagnosis	Duration (years)	Treatment*	Rose-Waaler titre	[C-reactive protein] (mg/l)
1	M	65	RA	5	4	>4096	6
2	M	65	RA	13	4	Negative	24
3	M	42	RA	7	3	64	24
4	F	65	RA	0·5	2	256	24
5	F	57	RA	38	2	4096	12
6	F	71	RA/OA	30	4	Negative	ND
7	F	74	RA	30	3	64	6
8	F	51	RA	30	3	4096	12
9	M	23	Trauma	—	1	ND	ND
10	M	42	Trauma	—	1	Negative	<6
11	M	59	OA	20	2	Negative	<6
12	M	71	OA	1	2	Negative	<6
13	F	50	PA	5	2	Negative	ND
14	F	24	PA	4	1	Negative	<6
15	F	30	PA	5	2	Negative	<6
16	F	29	Monoarthritis	0·1	2	Negative	ND

RA, definite or classical rheumatoid arthritis (ARA criteria); OA, osteoarthritis; PA, psoriatic arthritis; ND, not done.

* 1, no treatment; 2, non-steroidal anti-inflammatory drug only; 3, low-dose prednisolone (< 7·5 mg/day) or sulphasalazine; 4, slow-acting rheumatoid drug.

indicated, and bone marrow samples were obtained from patients with no significant haematological disorder in accordance with protocols accepted by the Ethics Committee of The Walter and Eliza Hall Institute. Clinical details of the patients described in this study are shown in Table 1.

Samples

Synovial fluid was collected into Vacutainers (Becton Dickinson, Rutherford, NJ) containing preservative-free heparin and incubated for 10 min at 37°C with hyaluronidase (*Streptomyces hyaluronlyticus;* Calbiochem, La Jolla, CA) at a final concentration of 1 turbidity reducing unit/ml. After pelleting the cells, the fluid was filtered and stored at −20°C in fractions. Sera obtained at the same time were also stored at −20°C. The C-reactive protein estimations were performed using the CRP-Wellcotest (Wellcome, Beckenham, UK). In some cases, 2 ml samples of synovial fluid or serum were dialysed against distilled water for 48 h at 4°C in order to precipitate possible inhibitors of colony formation as previously demonstrated (Chan, Metcalf & Stanley, 1971; Metcalf *et al.*, 1971).

Colony-stimulating factor preparations

The standard source of semi-purified human GM-CSF (CSF-α) and G-CSF (CSF-β) was medium conditioned by the bladder carcinoma cell line, U5637 (bladder CM), fractionated on a phenyl-Sepharose column and provided by Dr N.A. Nicola (WEHI, Melbourne). Partially-purified, Stage II HU-CSF was provided by Dr A.W. Burgess (Ludwig Institute, Melbourne). L cell conditioned medium (LCCM), a source of murine M-CSF was provided by Dr J.A. Hamilton (Department of Medicine, Melbourne University) and purified murine G-CSF was donated by Dr N.A. Nicola. The control stimuli did not display high-dose inhibition and, unless otherwise stated, were used at concentrations found to be maximal in the colony-forming assay.

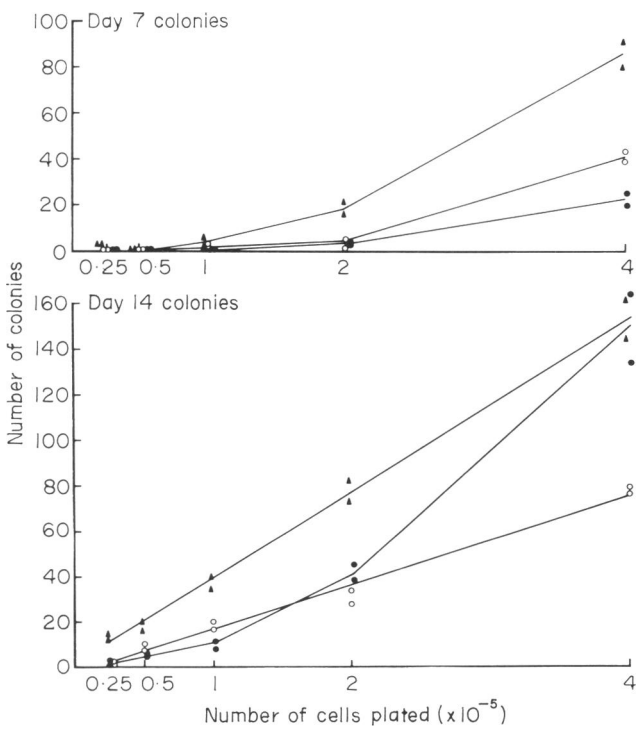

Fig. 1. Relationship of colony number to number of human bone marrow cells plated in cultures stimulated by synovial fluid. Duplicate cultures containing the indicated number of light-density cells were incubated for up to 14 days in the presence of either saline (○), or synovial fluid from patients with rheumatoid arthritis (▲) or osteoarthritis (●).

Table 2. Morphology of murine day 7 and human day 14 colonies stimulated by synovial fluid

Experiment number	Bone marrow source*	Stimulus†	No of colonies per 10^5 cells	Colony morphology (%)			
				Eo‡	G	GM	M
I	Human	Human GM-CSF	12	3	0	6	91
		Human G-CSF	38	0	33	13	54
		SF 7	18	0	4	4	92
II	Human	U5637 C.M.	38	13	26	34	27
		SF 7	38	14	32	25	29
		SF 16	29	7	41	20	32
III	Human	Human GM-CSF	73	15	39	19	27
		Human G-CSF	66	0	64	17	19
		SF 2	59	0	30	21	49
		#35-39	46	0	51	30	19
IV	Murine	Human G-CSF	74	0	58	38	4
		Murine G-CSF	60	0	55	44	1
		SF 2	170	0	4	21	75
		#35-39	114	0	17	42	41
V	Murine	Human G-CSF	49	0	40	47	13
		Murine G-CSF	44	0	57	32	11
		Human urinary CSF	215	0	7	14	79
		Murine L cell C.M.	234	0	4	16	80
		#31-34	62	0	3	13	84
VI	Murine	Human G-CSF	16	0	91	9	0
		Murine G-CSF	9	0	88	12	0
		Human urinary CSF	74	0	12	20	68
		Murine L Cell C.M.	120	0	8	30	62
		SF 5	32	0	2	10	88
		SF 6	41	0	5	19	76

* Human unfractionated (II), light-density (III) or light density, non-adherent (I) bone marrow cells were plated in triplicate at 1×10^5, 3×10^4 or 5×10^4 cells/ml respectively. Unfractionated murine bone marrow cells were plated in duplicate at $7{\cdot}5 \times 10^4$ cells/ml.

† Concentrations of standard stimuli (not necessarily maximal) were chosen to afford comparison of morphology in plates of similar colony density.

‡ Murine eosinophil colonies were seen in control plates stimulated with a source of murine Multi-CSF and Eo-CSF (data not shown).

Colony-forming assays

Human and murine (C57BL) agar cultures were prepared as described previously (Metcalf, 1984). Human bone marrow cells were washed and the light-density fraction was obtained after centrifugation on Ficoll-Paque (1·077 g/ml; Pharmacia, Uppsala, Sweden) for 20 min at 1000 ***g***. In some experiments, the non-adherent fraction was obtained after incubating the interface cells in a polystyrene flask (Lux 5350, Miles Laboratories, Naperville, IL) for 1 h at 37°C.

Stimuli (0·1 ml) were placed in 35 mm Petri dishes (Kayline Plastics, Adelaide, Australia) to which were added $3\text{–}10 \times 10^4$ cells in 1 ml agar mixture. Duplicate or triplicate cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2 in air for up to 14 days. Colonies were counted using a dissection microscope, then the whole plates were fixed with 2·5% glutaraldehyde, stained with luxol fast blue/haematoxylin, and the colony morphology was classified at ×400 magnification. The concentration of stimulus inducing the growth of 50% maximal colonies was defined as 50 U/ml. The presence of inhibitors of human colony formation was assessed by mixing 0·1 ml undiluted synovial fluid with an equal volume of bladder CM at different concentrations.

Murine WEHI-3B(D^+) differentiation assay

The BALB/c myelomonocytic leukaemia cell line, WEHI-3B(D^+), forms undispersed colonies of blast cells in unstimulated semi-solid agar cultures, but differentiates to form dispersed colonies containing mature monocytes and neutrophils if stimulated with either murine or human G-CSF (Nicola, Begley & Metcalf, 1985). Stimuli (0·1 ml) were added to 1 ml semi-solid agar cultures containing 300 WEHI-3B(D^+) cells. The proportion of differentiated colonies was scored after 7 days incubation.

Phenyl-Sepharose fractionation

The fractionation of bladder CM using phenyl-Sepharose chromatography has been described by Nicola (Nicola, Begley & Metcalf, 1985). Synovial fluid samples from three patients were made up to 1 M in ammonium sulphate and applied to a column of phenyl-Sepharose CL-4B (2·6 × 20 cm) equilibrated in 1 M ammonium sulphate/0·1 M sodium phosphate buffer, pH 6·0, containing Tween 20 (0·02%) and sodium azide (0·02%). The column was eluted with a 200 ml linear gradient from the initial buffer to distilled water, followed by a 200 ml linear gradient to 75% ethylene glycol at a flow rate of 12 ml/h.

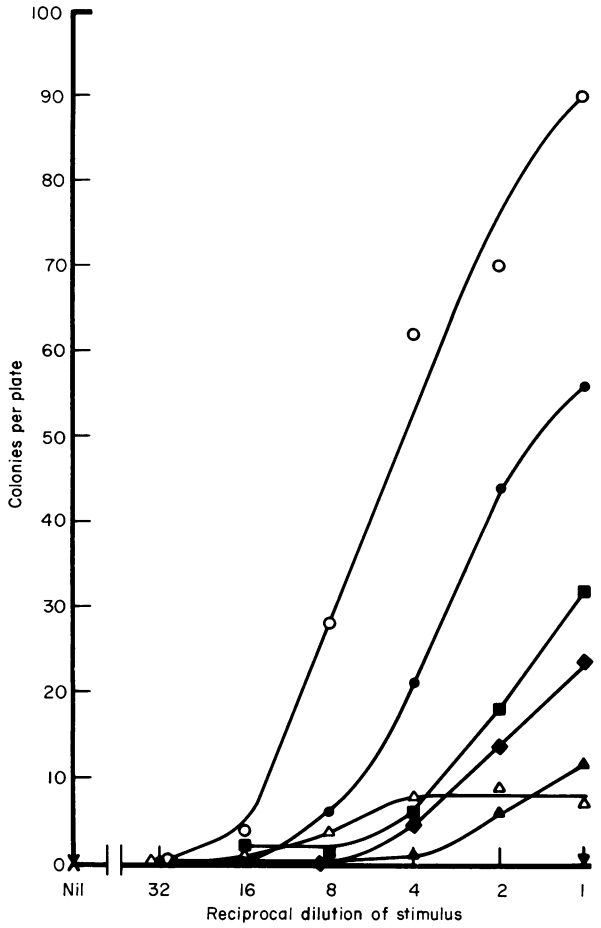

Fig. 2. Stimulation of murine colony formation by synovial fluid. $7{\cdot}5 \times 10^4$ unfractionated bone marrow cells were added to plates containing either medium (×), GM-CSF (▼), G-CSF (▲), murine G-CSF (△), HU-CSF (●), L cell CM (○) or synovial fluid (2■; 5♦). Colonies were counted after 7 days incubation.

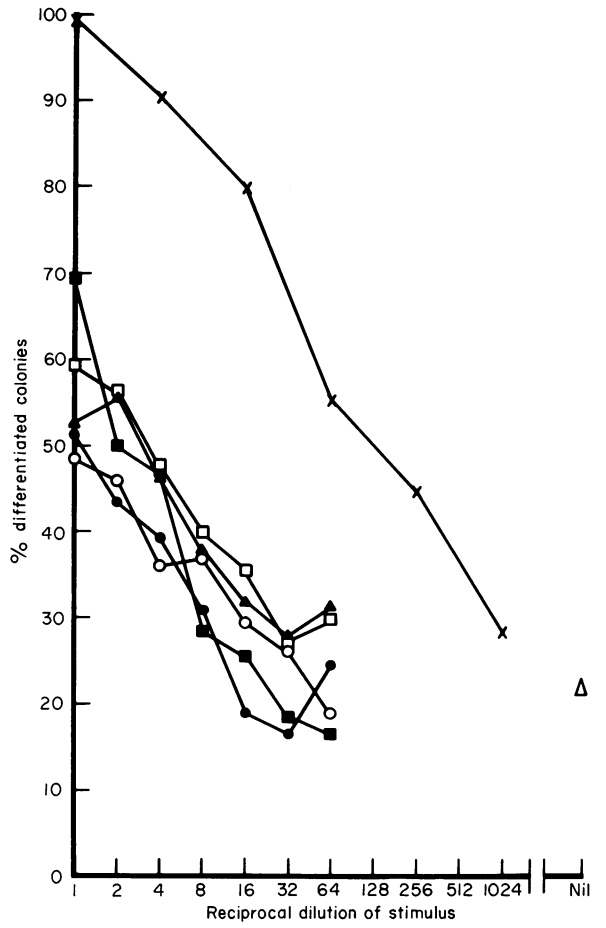

Fig. 3. Induction of differentiation in WEHI-3B(D^+) cells by synovial fluid. The percentage of differentiated colonies was scored after 300 cells had been cultured for 7 days in the presence of either saline (△), murine G-CSF (×) or synovial fluid from Patients 2 (○ ■), 13 (●), 14 (▲) or 15 (□).

Fractions (4 ml) were collected, exchanged into phosphate-buffered (20 mM, pH 7·4) saline (0·15 M) containing Tween 20 (0·02%) by passage through a PD10 column, sterilized (0·45 μm filters; Millipore) and assayed on mouse and human bone marrow. Active fractions were pooled, concentrated by ultrafiltration, exchanged and sterilized.

RESULTS

Detection of human colony-stimulating activity

Synovial fluid samples from seven of the first 14 patients listed (Table 1), on which all of the assays were performed, stimulated the growth of human haemopoietic colonies. Comparison with standard titrations of GM-CSF revealed that activities of up to 500 U/ml were detectable (95% prediction interval = 120–2500). A further three samples showed equivocal colony-stimulating activity and in four it was undetectable. Growth of the clones to colony size in low-density cultures was apparent only after 14 days incubation and at this time the number of colonies stimulated by RA synovial fluid was linearly related to the number of cells plated (Fig. 1). In the presence of colony crowding, a situation in which stimuli are more likely to mediate indirect effects via accessory cells, RA synovial fluid stimulated the growth of day 7 colonies and an otherwise inactive sample from a patient with osteoarthritis induced day 14 colonies. The morphology of the day 14 colonies stimulated by synovial fluid is shown in Table 2 (I–III). Sample 7 stimulated proportions of neutrophil (G), neutrophil-macrophage (GM) and macrophage (M) colonies similar to GM-CSF, the predominant factor in bladder CM inducing day 14 colonies.

The direct action of RA synovial fluid on the progenitor cells was demonstrated by transferring individual clones initiated in a standard preparation of CSF to a second dish containing synovial fluid. After a further 5 days incubation, 16/25 clones transferred to synovial fluid had more than doubled in size compared to 10/24 and 5/38 clones transferred to CSF or medium respectively (χ^2 $P < 0{\cdot}01$).

It was possible that the absence of detectable colony-stimulating activity in certain synovial fluid samples was due to the presence of inhibitors. Sample 3 was shown to have inhibitory activity, and it may be relevant that this was the only patient who had received an intra-articular steroid injection within 2 weeks of the sample being taken. Samples 9 and 11 were also found to contain inhibitors.

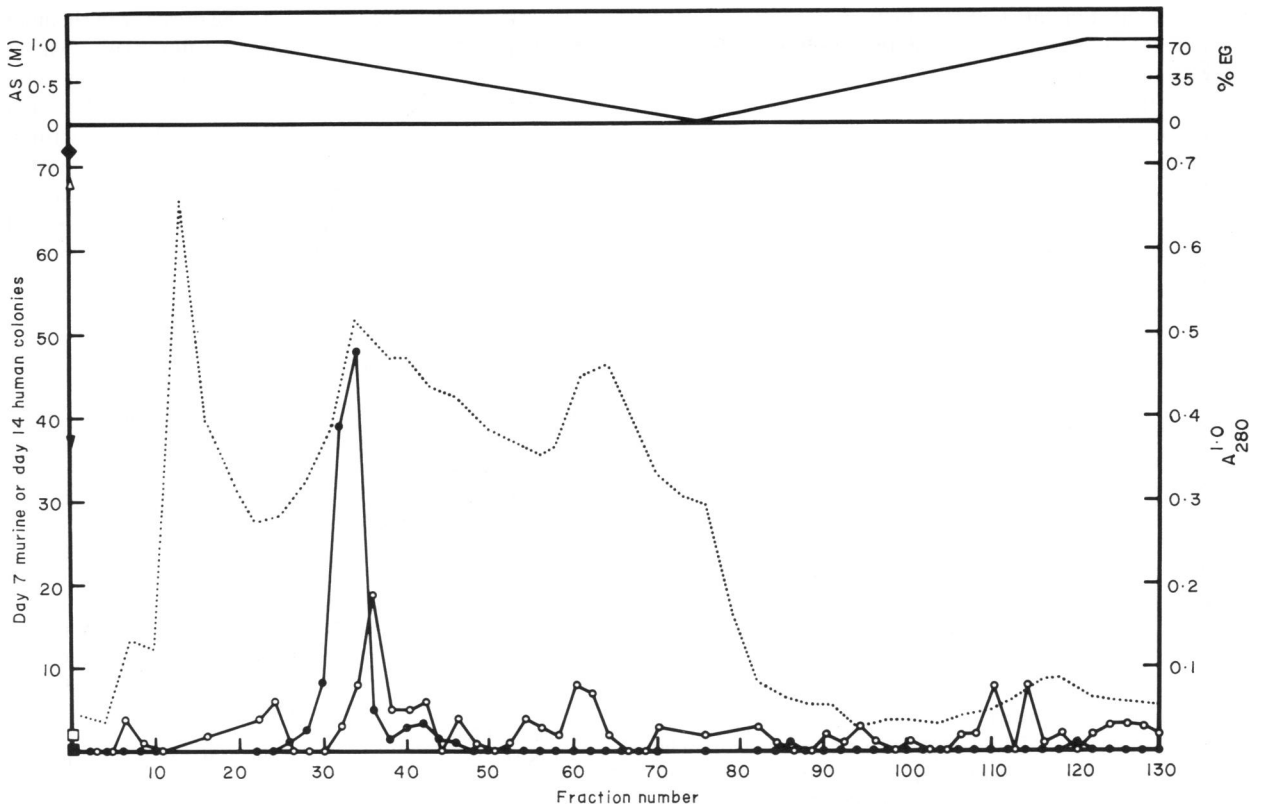

Fig. 4. Phenyl-Sepharose fractionation of synovial fluid from Patient 2. Samples of the fractions (0·2 ml or 0·1 ml respectively) were assayed on unfractionated human (○) or murine (●) bone marrow cells cultured at $7·5 \times 10^4$ cells per plate. Control stimuli: saline (□, ■), GM-CSF (△), G-CSF (▼) and HU-CSF (♦), the last being used at a half-maximal concentration. (. . .) $A^{1·0}_{280}$.

Detection of murine colony-stimulating activity

All of the samples which stimulated human colony formation also stimulated murine colonies, and in addition some samples only had detectable murine activity. A titration of two samples is shown in Fig. 2 and the morphology of the colonies stimulated in representative experiments is shown in Table 2 (IV–VI). The relative proportion of GM colonies stimulated varied between samples, but in some cases it closely resembled the proportions which arose in response to HU-CSF (VI).

In a limited number of cases in which colony-stimulating activity had already been demonstrated, the human and murine colony-stimulating activity of the synovial fluid was compared with that of a paired serum sample. With or without preliminary dialysis of the matched samples to remove potential inhibitors, the activity, although low, was greater in the synovial fluid than in the serum (data not shown).

Detection of WEHI-3B (D^+) differentiation-inducing activity

Typical titrations of active synovial fluid samples in one experiment are shown in Fig. 3. With two exceptions, all of the samples which stimulated murine colony growth also induced WEHI-3B (D^+) differentiation. One of these inactive samples (No. 5) resembled sample 6 and HU-CSF in stimulating predominantly murine macrophage colonies, but the latter sample was also active on WEHI-3B (D^+) which would be atypical of HU-CSF. Therefore there was no clear correlation between the ability of a sample to stimulate a high proportion of murine granulocyte colonies and its ability to induce WEHI-3B (D^+) differentiation. Two (samples 3 and 9) of the three samples containing inhibitors of human colony formation were toxic to WEHI-3B (D^+) cells and the third (sample 11) was inactive.

Phenyl-Sepharose chromatography of synovial fluid

Since hydrophobic chromatography of bladder CM had been found to successfully separate GM-CSF and G-CSF, 30 ml of synovial fluid from Patient 2 was fractionated on a phenyl-Sepharose column and assayed on mouse and human bone marrow (Fig. 4). The active fractions were relatively hydrophilic and eluted where one would expect to find GM-CSF, however their action on murine cells was more typical of HU-CSF (Table 2, Expt V). Although no WEHI-3B (D^+) differentiation-inducing activity was detectable in the individual fractions, when they were pooled and concentrated in groups of 10, more was found in fractions 21–30 than in 31–40 (data not shown).

DISCUSSION

This paper reports the detection of colony-stimulating factors in the synovial fluid of patients with various inflammatory arthropathies. This is supported by the following evidence:

(a) The linear relationship between the number of cells plated and the number of day 14 colonies stimulated. There was also a suggestion from the non-linear response of day 7 colonies that additional factors were present in the rheumatoid synovial

fluid which could stimulate CSF production by accessory cells. This could be important in view of the implication from a comparison of serum and synovial fluid CSF levels (data not shown) that the CSF may be produced locally. Interleukin 1 (also known as hemopoietin 1) has been detected in synovial fluid (Fontana *et al.*, 1982; Wood *et al.*, 1983), can stimulate production of CSF (Sieff, Tsai & Faller, 1987; Seelentag *et al.*, 1987) and can act synergistically with CSF to enhance murine colony formation (Stanley *et al.*, 1986). However, it does not itself stimulate colony formation and its presence in synovial fluid could not account for the results presented here.

(b) The ability of synovial fluid to maintain the proliferation of individual clones, free of accessory cells, which had been initiated in CSF.

(c) The ability of synovial fluid to initiate the proliferation of fractionated human promyelocytes (Begley *et al.*, 1985) at low cell density (data not shown). This also indicated that the putative CSF could act on cells of the myeloid series at a relatively late stage of differentiation which is important because the colony-forming experiments suggested that early (day 14) progenitors were preferentially stimulated by synovial fluid. There was no evidence of selective inhibition of day 7 colonies; on the contrary, there seemed to be a synergistic action of synovial fluid and bladder conditioned medium on these late progenitors (data not shown).

It is likely that synovial fluid contains a mixture of CSF. The development of eosinophil colonies and the relative proportions of granulocyte-macrophage colonies in the human marrow cultures suggested the presence of GM-CSF. At present there is no specific assay for Multi-CSF but the detection of mast cells in the rheumatoid synovium (Crisp *et al.*, 1984; Godfrey *et al.*, 1984) suggests that it may also be important. The stimulation of murine colonies of predominantly monocyte-macrophage morphology resembled the action of M-CSF (HU-CSF, CSF-1) and the induction of WEHI-3B(D^+) differentiation was typical of G-CSF. Hydrophobic chromatography failed to separate these activities definitively but did resolve at least one major peak of activity. In summary, the full range of activities exhibited by the synovial fluid samples cannot be ascribed to any one of the known human CSF acting in isolation so efforts must be directed towards further fractionation procedures. Unfortunately these may be hampered by the restricted availability of material and the heterogeneous behavior of different synovial fluid samples.

Although not intended as a comparative clinical study of inflammatory and non-inflammatory arthropathies, the data from these patients, and a limited number of others, suggest that the presence of CSF may be related to disease activity. Of 12 patients with 'inflammatory arthropathies' (rheumatoid or psoriatic arthritis), 11 had detectable murine colony-stimulating and/or differentiation-inducing activity compared with one out of four controls (osteoarthritis, trauma). Larger studies must await the development of sensitive immunoassays because the bioassays are variable and only semiquantitative. However, this study using bioassays is an essential prerequisite to confirm that immunoactivity can be related to biological activity, particularly in samples which may contain proteases.

The stimulation of granulocyte and macrophage function *in vitro* by low concentrations of CSF and the enhancement of macrophage phagocytic capacity by CSF administration *in vivo*, suggest that activation of mature cells may be an important property of these molecules *in vivo*. The presence in rheumatoid synovium of T cells, macrophages and fibroblasts, which are known sources of CSF, their close proximity to targets of CSF action, for example macrophages, neutrophils and mast cells, and finally the detection of colony-stimulating factor(s) in synovial fluid support the hypothesis that CSF are important in the pathogenesis of this and other inflammatory diseases. The development of antagonists of CSF production and/or action for local use in diseases such as RA may therefore be appropriate.

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