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Interleukin 1 Activity in the Synovial Fluid of Patients with Rheumatoid Arthritis

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Summary. The synovial fluids (SF) of patients with rheumatoid arthritis (RA) were investigated for their effects on thymocytes of C3H/HeJ mice. Of the 20 SF tested, 17 (85%) showed an augmentation of the phytohaemagglutinin (PHA) induced thymocyte stimulation. Out of 16 SF of patients with osteoarthrosis, such an activity was detected in only one (6.25%). Further characterisation of the amplification factor revealed that (1) the SF of RA patients augmented both the PHA and the Concanavalin A response of the thymocytes (2) in the absence of mitogens, SF-treated thymocytes showed an increased uptake of ^{3}H-thymidine, (3) the SF did not propagate the growth of an interleukin 2 dependent ovalbumin specific T cell clone, but (4) the SF were found to be required for optimal interleukin 2 release by spleen cells stimulated with suboptimal doses of lectin. Based on these biological effects the factor in the SF of RA patients is suggested to represent an interleukin 1 (IL-1). IL-1 produced in cultures by activated macrophages has been shown to stimulate T and B cell functions and to induce the production of collagenase and prostaglandins by cultured synovial cells. Both properties of IL-1 could be relevant in the pathogenesis of RA.

Key words: Rheumatoid arthritis – Lymphocyte activating factor – Interleukin 1 – T cell growth factor – Interleukin 2 – Mitogenic factor – Macrophages

Introduction

In rheumatoid arthritis (RA) genetically controlled immunological processes appear to be important factors in the initiation and perpetuation of the inflammatory events within the synovial tissue of the joints. Increased numbers of activated lymphocytes can be found in the peripheral blood, synovial membrane (SM), and synovial fluid (SF) of patients with RA. This has been shown both by morphological studies and by functional assays measuring the spontaneous proliferation of lymphocytes of SM and rheumatoid SF [1–3]. Additional evidence for the in vivo activation of lymphocytes is provided by the finding of migration inhibition factor (MIF) production [4] and immunglobulin secretion [5] by the rheumatoid SM. The signals leading to the activation of lymphocytes in RA are poorly understood. Aggregated IgG and IgG in immune complexes [6] as well as tissue degradation products such as collagen [7] and proteoglycans [8] may amplify the ongoing immune response. Furthermore an unspecific mitogenic factor (MF) was found in the SF of RA [4]. This observation prompted us to characterize further the MF detected. Our experiments demonstrate that an interleukin 1 (IL-1) like activity is present in the SF of RA patients, which promotes the production of T cell derived T cell growth factor (TCGF) or interleukin 2 (IL-2).

Materials and Methods

Selection of Patients and Preparation of SF. Patients with RA were classified by using the criteria of the American Rheumatism Association [9]. Only those patients with definite and classical RA and in addition patients with osteoarthrosis (OA) were selected, which had low concentrations of lactatdehydrogenase (LDH, < 0.5 U/ml) and no measurable activity of collagenase and leukozyte elastase in the SF [10]. In the selected SF, only a low concentration of LDH levels was observed after centrifugation of the SF cells and after freezing the supernatant. This excludes a significant release of intracellular molecules leaking out of in vitro damaged cells. Before testing the SF for MF activity, the fluids were diluted 1 : 10, passed through two filters (0.45-μ and 0.20-μ (Gelman, Ann Arbor, Michigan, USA)) and applied on a micropartition system (MPS-1 with YMB membrane, Amicon Corporation, Danvers, Massachusetts, USA) in order to remove low molecular weight material (mol. wt. < 10 000).

Production of Human IL-1 and IL-2. Peripheral blood mononuclear leukocytes (PBM) of normal blood donors were isolated by Ficoll-Hypaque density sedimentation. For *IL-2 production* 5×10^6 PBM were incubated in plastic tubes (Falcon, Cat. No. 3033, Div. Becton, Dickinson and Co, Oxnard, California, USA) containing 1 ml of RPMI 1640 medium supplemented with 1% autologous human serum, 300 μg L-glutamin, 50 U penicillin, 50 μg streptomycin and 5 μg Concanavalin A (ConA, Pharmacia Fine Chemicals, Uppsala, Sweden). After 48 h of culture, the PBM were removed by centrifugation ($2000 \times g$, 10 min), and the

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supernatant (SN) was passed through a 0.45-μ filter. For *IL-1 production* adherent cells of PBM from healthy blood donors were prepared by incubating a suspension of 1×10^7/ml PBM at 37 °C for 4 h in petri dishes (Falcon, Cat. No. 3001F). Thereafter, the SN containing non-adherent cells were decanted. The resultant adherent PBM were washed three times and incubated for 48 h in the presence of lipopolysaccharide (10 μg/ml, LPS of *E. coli* 0127: B8, Difco Laboratories, Detroit, Michigan, USA) and indomethacin (1 μg/ml, Sigma Chemical Company, St. Louis, Missouri, USA). The cell SN was centrifuged ($2000 \times g$, 10 min) and passed through a 0.45-μ filter. These IL-1 and IL-2 preparations were used as standards.

Production of Mouse IL-2. In experiments performed to monitor IL-2 production by C3H/HeJ mouse spleen cells upon stimulation with SF or a human IL-1 standard, 5×10^6 spleen cells were incubated in RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol (2-ME), 300 μg/ml L-glutamin, 50 U/ml penicillin, 50 μg/ml streptomycin and ConA (1 μg/ml or 5 μg/ml). The SF or IL-1 standard was added to the spleen cells to give a final concentration of 1 : 15. At the conclusion of the culture periods, the cells were removed by centrifugation ($1000 \times g$, 10 min) and the SN assayed for IL-2 activity.

Assay for IL-1 and IL-2 Activity. The *IL-1 activity* of SF was determined by measuring the augmenting effect on phytohaemagglutinin (PHA, Wellcome Research Lab., Beckenham, England) stimulated thymocytes. The assays were performed in microculture plates with flat-bottom wells by culturing 600 000 thymocytes of C3H/HeJ mice in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 300 μg/ml L-glutamin, 1×10^{-5} M 2-ME, 50 U/ml penicillin, 50 μg/ml streptomycin and PHA (0.5 μg/culture well). The SF were added at a final concentration of 1 : 20. Cultures were done in quadruplicate and incubated for 72 h at 37 °C in a 5% CO_2-in-air incubator. The rate of DNA synthesis was determined by measuring the incorporation of ^{3}H-thymidine (^{3}H-Tdr, 0.5 μCi added per well, specific activity 2 Ci/mmol, New England Nuclear, Dreieich, FRG) after a 16 h incubation of the nucleotide. The effect of SF on the PHA response of thymocytes was compared with the human IL-1 standard (1 U/ml).

The *IL-2 activity* was assayed using a strictly IL-2 dependent cloned ovalbumin specific mouse T cell as the indicator cells; 10000 cells per well were incubated for 48 h in Iscove's modified DMEM (Gibco Bio-Cult Ltd., Paisley, Scotland) supplemented with 100 U/ml penicillin and streptomycin, 5×10^{-5} M 2-ME and 10% FCS. The incorporation of ^{3}H-Tdr was measured over a 12 h period.

Results

In experiments designed to test the effect of SF on the PHA response of C3H/HeJ thymocytes, the results of the SF were expressed as U/ml of activity based on a standard human IL-1 preparation. With the IL-1 standard obtained by taking the SN of LPS stimulated human adherent mononuclear cells, generally a 15- to 20-fold increase of the PHA response of thmyocytes was observed. Compared with the effect of the IL-1 standard (1 U/ml), 17 of 20 SF (85%) of patients with RA showed a significant activity on the PHA response of thymocytes (> 0.15 U/ml). In contrast, in all but one of the SF of patients with OA no such activity was detected: 1 SF of 16 SF tested was positive (6.25%) (Fig. 1). These SF had an average activity of 0.07 ± 0.04 U/ml, whereas the corresponding value in the RA group was 0.48 ± 0.32 U/ml.

In patients with RA no direct correlation was found between the SF activity on thymocytes and the amount of rheumatoid factors, C-reactive protein, complement levels (C3, C4), and blood sedimentation rate. The study of the clinical history or the treatment schedule of the patients was likewise unrevealing (data not shown).

Experiments were designed to evaluate the nature of the modulatory effects of SF on mouse thymocytes. The tests were performed by using four selected SF (nos. 168, 183, 211 and 227), which showed various degrees of activity on the PHA response of thymocytes. The SF were found to augment not only the PHA but also the ConA stimulation (Table 1). Compared with untreated cell cultures, thymocytes showed an up to tenfold increase in the ^{3}H-tdr incorporation when incubated for 72 h in the presence of SF. Evidence for the presence of inhibitory factors in SF was obtained by testing different dilutions of SF or not ultrafiltrated SF. The SF diluted 1 : 4 or 1 : 8 were less active in augmenting the PHA response of thymocytes than SF diluted 1 : 32 (Fig. 2 A). The effect of the SF on ConA-stimulated thymocytes became only apparent after ultrafiltration of the SF (Fig. 2 B).

The evidence that the augmentation of the PHA response of thymocytes was not mediated by IL-2 was obtained when the SF were tested on target cells insensitive to IL-1 but sensitive to IL-2. These cells grew only in

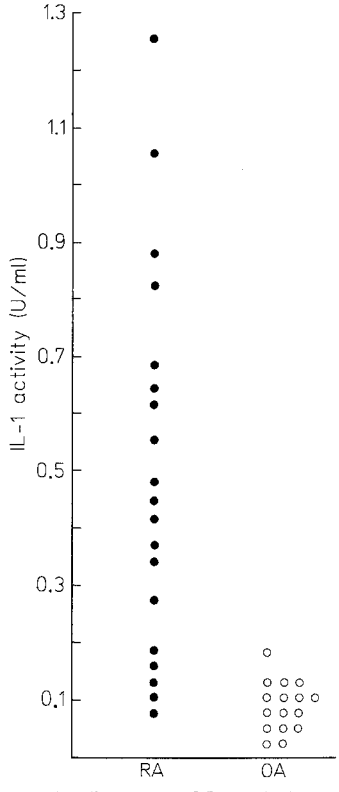

Fig. 1. The cpm of ^{3}H-Tdr incorporated in cultures in the presence of PHA and a human IL-1 standard (1 U/ml) was compared with the cpm of thymocyte cultures treated with PHA and ultrafiltrated SF (final dilution 1 : 20) of patients with rheumatoid arthritis (*RA*) or osteoarthrosis (*OA*). The data are given as IL-1 U/ml

Table 1. Effect of SF of patients with RA on mouse thymocytes

Stimulant added to thymocytes	Dose (μg/ml)	^{3}H-Tdr incorporation (cpm) ± SD by thymocytes incubated with				
		Medium control	Synovial fluids (sample number)[a]			
			168	183	211	227
None	0	513± 67	4 022± 332	5 613± 467	2 118± 112	1 026± 103
ConA	1	70 212±4 059	149 077±3 304	120 828±6 931	109 321±2 738	96 767±2 217
PHA	0.5	4 622± 668	84 295±4 359	64 333±6 932	34 183±3 283	21 305± 368

[a] The ultrafiltrated SF were added to the thymocyte cultures to give a final dilution of 1 : 20

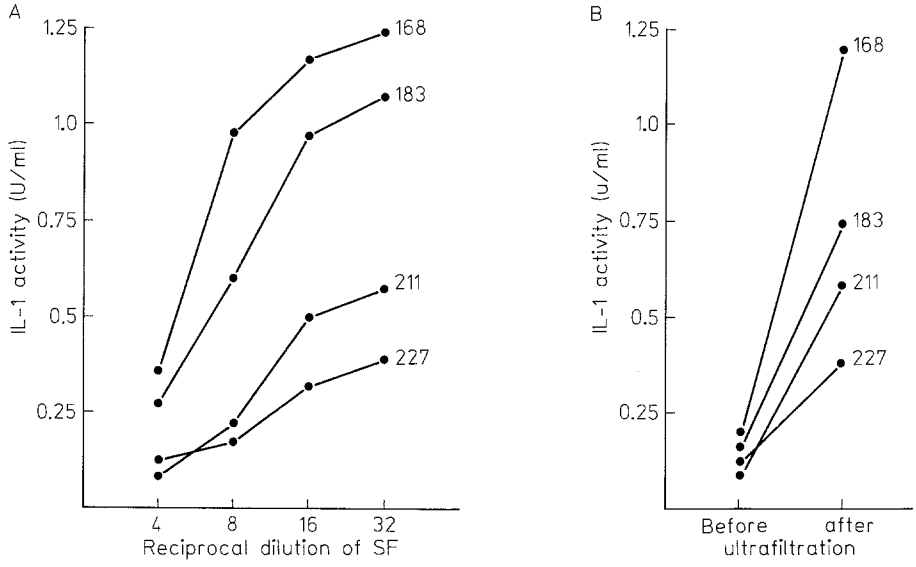

Fig. 2 A, B. The activity of various dilutions of ultrafiltrated SF were tested on PHA stimulated thymocytes (**A**) whereas the effect of ultrafiltration on the activity in SF (final dilution 1 : 20) was investigated on ConA stimulated thymocytes (**B**). The SF of four RA patients were tested (nos. 168, 183, 211 and 227)

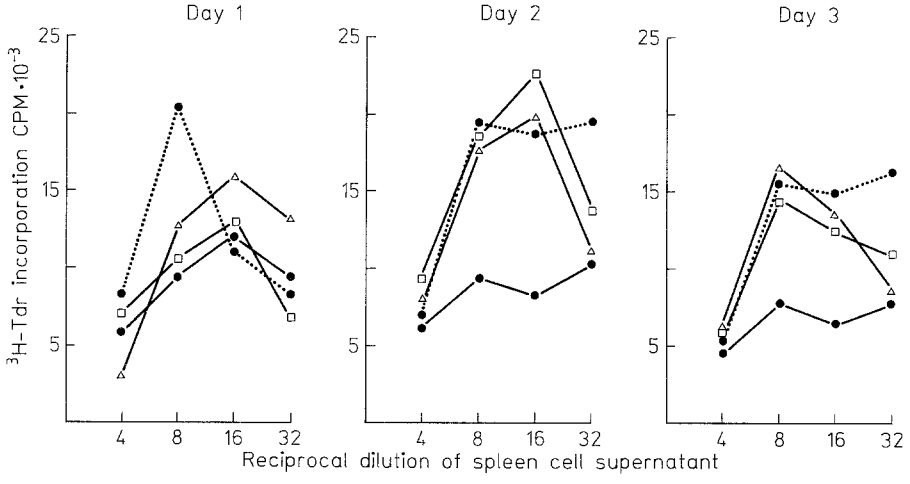

Fig. 3. Spleen cells of C3H/HeJ mice were stimulated with ConA 5 μg/ml (. . . . .) or ConA 1 μg/ml (——). The cultures treated with suboptimal doses of ConA (1 μg/ml) were supplemented with either the human IL-1 standard (□) or the ultrafiltrated SF of the RA patient no. 168 (△), or with a medium control (●). The data are expressed as cpm of ^{3}H-Tdr incorporated in ova cells treated with various dilutions of SN of spleen cell cultures collected after 1, 2 and 3 days

Table 2. Effects of SF on IL-2 dependent and independent cells

Culture medium supplemented with[a]	^{3}H-Tdr incorporation (cpm) ± SD in cultures of	
	PHA stimulated thymocytes	Ova cells[b]
None	6 367± 658	534± 107
IL-1 standard	72 026±1 144	309± 25
IL-2 standard	84 117±7 381	47 562±4 028
SF no. 168	78 510±5 250	302± 17
SF no. 183	66 793±6 747	377± 63
SF no. 211	54 495±7 580	186± 37
SF no. 227	37 133±2 457	548± 87

[a] Final dilution 1 : 15
[b] Cloned ovalbumin specific mouse T cells

the presence of the human IL-2 standard but not in the presence of the four SF (Table 2).

The suggestion of an IL-1-like factor in the SF was substantiated when testing the SF for their capacity to stimulate spleen cells to release IL-2. Conditioned media, prepared from spleen cells stimulated by ConA, contained IL-2. The result presented in Fig. 3 show an accumulation of IL-2 during the first 48 h in SN of ConA-stimulated spleen cell cultures. The activity of the SN was ConA-dose dependent. A stimulation of spleen cells with 5 μg/ml ConA provided more active SN then a stimulation with 1 μg/ml ConA. However, peak levels of IL-2 activity were found in spleen cell cultures treated with both a suboptimal ConA concentration (1 μg/ml) and SF. This dual effect of SF and lectin on IL-2 production was observed when testing 1 : 8 and 1 : 16 dilutions of SN of spleen cells cultured for 48 and 72 h. When adding, instead of SF, the IL-1 standard to spleen cells stimulated with ConA (1 μg/ml), the same results were obtained. Higher IL-2 activities were found in 1 : 32 diluted SN of spleen cells stimulated with ConA 5 μg/ml compared with the same dilutions of spleen cells stimulated with ConA 1 μg/ml in the presence of the SF or the IL-1 standard (Fig. 3).

Discussion

The requirement of macrophages for T lymphocyte activation by antigens, mitogens and allogeneic cells has repeatedly been demonstrated. Macrophages are also required for B lymphocyte activation by soluble protein antigens and T cell dependent antigens [for review see 11]. In addition, macrophages were shown to play an important role in the unspecific activation of lymphocytes. When antigen- or mitogen-activated T cells, possibly helper T cells, interact with a soluble macrophage derived factor (IL-1 or lymphocyte activating factor), they will release TCGF (IL-2) [12]. The latter is required by lymphocytes to proliferate. One of the most convincing arguments for an in vivo role of immune regulatory factors is their demonstration at the sites of immune responses. The only report to date published on the presence of helper factors in vivo is the finding by Stastny et al. [4] of an unspecific mitogenic factor in the SF of patients with RA. The activity was not detected in patients with OA [4].

In the present study an IL-1-like activity was found in the SF of patients with RA. The factor detected shares the biological characteristics of IL-1 released in culture by activated macrophages: (1) the SF of RA patients augment the PHA and ConA response of mouse thymocytes; (2) SF treated thymocytes show an increased ^{3}H-Tdr uptake compared with untreated cells even in the absence of mitogens; (3) the SF factor does not support the growth of a IL-2 dependent T cell clone; (4) together with suboptimal doses of ConA the factor was found to be required for optimal IL-2 release. The augmentation of the ConA response of thymocytes by an SF factor was only observed when testing SF which has been ultrafiltrated to remove possible inhibitory proteins of low molecular weight. In addition, the effect of the ultrafiltrated SF on the PHA response of thymocytes only became visible by diluting the SF. Both observations show the presence of inhibitory factors, at least one having a molecular weight of less then 10 000. An inhibition of the immune response has been demonstrated by various cell products such as lymphocyte derived lymphotoxin or specific and unspecific suppressor factors as well as by macrophage products (e.g. prostaglandin). In the context of a viral hypothesis of RA, it may be important to point out that viruses can inhibit the activation of lymphocytes either directly, by stimulation of suppressor cells, or by triggering interferon release [13].

A comparative investigation of the biochemical nature of the factor responsible for the SF IL-1-like activity described here and the one detected in the SF by Stastny et al. [4] is warranted. It has been suggested that IL-1 and MF are produced by different cells, the IL-1 being a product of macrophages, the MF being secreted by T cells [for review see 14]. In view of the comparable experimental conditions used by Stastny et al. [4], or by us, both activities described might be due to identical molecules. It also has to be tested by biochemical analysis whether the factors in SF exerting IL-1-like effects are identical to IL-1 secreted by cultured macrophages in vitro.

As to the possible implication of our finding on the pathogenesis of RA the study demonstrating an IL-1 induced secretion of prostaglandin and collagenase by cultured human synovial cells is most interesting [15]. The presence of IL-1 in the SF in the absence of detectable collagenase could be due to the well-known fact that SF contains potent inhibitors of neutral proteinases including collagenase.

In RA the SM has many characteristics of a hyperactive immunologically stimulated lymphoid organ [16]. The loose connective tissue of the rheumatoid SM and the pannus, which leads to cartilage and bone destruction, is densely infiltrated with mononuclear cells. In immunhistological studies OKT_4^+T cells are found to form a close

contact to macrophage-like cells, the so-called dendritic or interdigitating HLA-DR$^+$ non-lymphoid cells. Within these HLA-DR$^+$/OKT$_4^+$ areas, B cells and plasma cells can be identified [16]. Although proof is lacking, the hypothesis is appealing that the IL-1 detected in SF may be secreted by activated macrophages (interdigitating cells?) within the SM and may have an in vivo activity on both collagenase production of synovial cells and activation of T-helper cells. These IL-1 activities could facilitate both tissue destruction by synovial cells and the development of T-effector cells as well as immunoglobulin secreting plasma cells.

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