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Interleukin-2 in rheumatoid arthritis: production of and response to interleukin-2 in rheumatoid synovial fluid, synovial tissue and peripheral blood

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SUMMARY

Several aspects of interleukin-2 (IL-2) generation and function were studied employing mononuclear cells from synovial fluid (SF), synovial tissue (ST) and peripheral blood (PB) of patients with rheumatoid arthritis (RA). Decreased PHA stimulated IL-2 production by lymphocytes from rheumatoid ST, SF ($P < 0{\cdot}02$), and PB ($P < 0{\cdot}01$) was observed when compared to normal blood and SF of patients with gout. The proliferative response of rheumatoid lymphocyte blasts exposed to exogenous IL-2 was also defective ($P < 0{\cdot}05$–$0{\cdot}001$). This defect was greater in SF than in rheumatoid PB ($P < 0{\cdot}05$–$0{\cdot}001$). In addition to the proliferative response, the effect of IL-2 on interferon-gamma (IFN-γ) production was also examined. Rheumatoid lymphocytes from both PB and SF produced less IFN-γ after overnight treatment with IL-2 than did normal PB lymphocytes. This decreased IFN-γ induction was discordant with the excellent enhancement by IL-2 of natural killer activity. Removal of adherent cells in synovial fluid did not correct this deficit. Abnormalities in the biology of IL-2 and IFN-γ suggest that impaired T cell function could contribute to the immunopathogenesis of RA.

Keywords interleukin-2 rheumatoid arthritis interferon-γ

INTRODUCTION

Interleukin-2 (IL-2), T cell growth factor, produced by activated helper T cells, is an important immunoregulatory molecule required for normal lymphocyte function (Farrar *et al.*, 1982). The synthesis of IL-2 leads to the production of interferon-gamma (IFN-γ) by T cells (Torres, Farrar & Johnson, 1982; Yamamoto, Farrar & Johnson, 1982; Kasahara *et al.*, 1983; Klein, & Bevan, 1983) and to the generation of natural killer (NK) cell activity (Handa *et al.*, 1983). Defects in the production of and response to IL-2 have been reported in autoimmune murine strains (Wofsy *et al.*, 1981; Dauphinee *et al.*, 1981; Altman *et al.*, 1981) and in human lupus erythematosus (Alcocer-Varela & Alarcon-Segovia, 1982; Linker-Israeli *et al.*, 1983).

Immune cells in the rheumatoid synovium are mainly T lymphocytes (Abrahamsen *et al.*, 1975). Their interactions with accessory cells are believed to play a major role in the dysregulation of the immune response in rheumatoid arthritis (RA) (Janossy *et al.*, 1981; Klareskog *et al.*, 1982).

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Synovial T lymphocytes have the characteristics of activated T cells (Galili, Rosenthal & Klein, 1981; Burmester *et al.*, 1981) and have been shown to be poor responders both to lectin stimulation (Panayi, 1973; Sheldon, Papamichail & Holborow, 1974; Burmester *et al.*, 1978) and to autologous stimulator cells in the autologous mixed lymphocyte reaction (Silver, Redelman & Zvaifler, 1983). In addition, T cells from RA patients have an impaired ability to regulate Epstein–Barr virus-induced B cell proliferation (Depper, Bluestein & Zvaifler, 1981). The mechanisms responsible for these abnormalities of T lymphocyte function in RA are poorly understood. In order to further characterize T lymphocyte function in RA, we studied production of and response to IL-2 employing T cells from synovial fluid (SF), synovial tissue (ST) and peripheral blood (PB) of patients with RA.

MATERIALS AND METHODS

Subjects. A total of 19 patients with definite or classical RA (Ropes *et al.*, 1958) were studied. SF was obtained from 15 patients and ST from four. Simultaneous PB samples were drawn from 15 of the 19 patients. The subjects (13 females and six males) had a mean age of 52 years (range 32–73). Seventeen patients were treated with NSAID, four with D-penicillamine, two with gold and three with low doses of corticosteroids ($< 7{\cdot}5$ mg/day). One patient was on no medications. None had a modification of treatment in the preceding 3 months. PB was also obtained from 15 healthy donors (nine females and six males) with a mean age of 48 years (range 30–65). In addition, SF from four patients with gout were studied.

Cell separation. PB mononuclear cells (MNC) were obtained from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Combe *et al.*, 1984; Miyasaka *et al.*, 1980) and washed three times in RPMI 1640 (KC Biological, Lenexa, Kansas, USA) containing 1% heat-inactivated fetal bovine serum (FBS) (KC Biological) and 10 mM HEPES. SF specimens were aspirated into heparinized syringes, incubated with hyaluranidase (50 μg/ml) (Sigma Chemical Co., St Louis, Missouri, USA, No. H2376) for 30 min at 37°C, and then handled as described above.

Separation of the cells from ST was performed as previously described (Burmester *et al.*, 1981; Combe *et al.*, 1984). Briefly, the specimens were minced into small pieces, mixed with RPMI 1640 containing 0·15 mg/ml DNAase (Sigma No. D4763), 0·5 mg/ml collagenase (Sigma No. CO130) and 50 μg/ml hyaluronidase. The specimens were stirred magnetically for 90–120 min at 37°C. The resultant cell suspensions were filtered through two nylon meshes with pore sizes of 250 μm and 88 μm (Tetco, Elmsford, New York, USA), washed twice and processed as described for PB. No interference of cellular function by different enzymes was observed when examined by comparing treated and untreated blood samples. The viability of cells was always greater than 95% by trypan blue exclusion.

Production of IL-2. MNC were suspended at 1×10^6/ml in 1% FBS complete RPMI (RPMI 1640 supplemented with L-glutamine, HEPES, penicillin, streptomycin and fungizone). Cell suspensions (2 ml) were incubated with and without 1 μg/ml of phytohemaglutinin (PHA) (Burroughs-Wellcome, Greenville, North Carolina, USA) in tissue culture plates (Costar No. 3506, Cambridge, Massachusetts, USA) for 24 h at 37°C. Supernatants were harvested after centrifugation, filtered through 0·45 μm filters (Millipore Corp., Bedford, Massachusetts) then stored at −70°C until the time of the assay.

IL-2 assay. IL-2 activity was detected using a murine IL-2-dependent cytotoxic T cell line (CTLL-2) kindly provided by Dr Steven Gillis (Immunex Corporation, Seattle, Washington, USA). This cell line was maintained in 10% FBS complete RPMI, 5×10^{-5}M mercaptoethanol and 10% IL-2. Human delectinated IL-2 from a single batch was purchased from Cellular Products, Inc. (Buffalo, New York) and will be referred as standard IL-2. Detection and quantification of IL-2 was performed as described by Gillis *et al.* (1978). Briefly, 100 μl of 5×10^4/ml CTLL cells in 10% FBS complete RPMI were placed in 96 well microplates (Falcon No. 3072, Becton–Dickinson, Oxnard, California, USA). Serial dilutions of the samples (100 μl) were added and the plates were incubated 24 h at 37°C. One microcurie of ^{3}H-thymidine (specific activity 6 Ci/mol. Schwarz–Mann, Orangeburg, New York) was then added for 4 h. The cultures were harvested on glass fibre filter

strips and counted on a liquid scintillation counter (Packard Instrument Co.). The incorporation of tritiated (^{3}H) thymidine by CTLL cells incubated with each tested sample was compared to the response with the standard IL-2 by probit analysis (Gillis *et al.*, 1978). Results are expressed as units of IL-2/ml (u/ml). One unit of IL-2 was arbitrarily defined, to allow for relative comparisons, as the reciprocal of the dilution of the sample tested, that gave a proliferative response equivalent to 10% of the maximal response generated by a highly enriched IL-2 standard. PHA by itself did not stimulate the CTLL cells.

Response to standard IL-2. Lymphocyte blasts were obtained after stimulation with Con A (10 μg/ml) and washed twice in 1% FBS-RPMI supplemented with 10 mg/ml of α-methylmannoside (Sigma). Ten thousand activated cells were cultured for 72 h at 37°C with several dilutions of the standard IL-2 in 10% FBS, complete RPMI containing 10 mg/ml of α-methylmannoside. The cells were pulsed with ^{3}H for the last 6 h and harvested.

NK assay. Effector cells (5×10^6) were incubated for 16 h at 37°C in 2 ml of 20% FBS-complete RPMI in culture plates (Costar No. 3506) with or without standard IL-2. The target for NK activity was K-562, a myelogenous leukaemia cell line. NK activity was quantitated in a 3 h ^{51}Cr release assay as previously described (Combe *et al.*, 1984; Seman *et al.*, 1981). Briefly, effector cells were mixed with labelled K-562 target cells at varying concentrations to give effector:target ratios of 20:1, 10:1 and 5:1. Triplicate samples were prepared for each ratio. The supernatants were withdrawn after a 3 h incubation period and the ^{51}Cr released from lysed targets was quantitated in a gamma counter (Packard Instrument Co.). Incubation of targets with medium or saponin (7 mg/ml) and EDTA (0·1 mg/ml) determined spontaneous and maximum release. The percent cytotoxicity was calculated by the formula:

$$\frac{(\text{ct/min of effector cells} - \text{ct/min of spontaneous release})}{(\text{ct min of maximum release} - \text{ct/min of spontaneous release})} \times 100.$$

IFN assay. The supernatants of cells cultured to determine NK activity with and without added IL-2 were collected after centrifugation and tested for IFN activity. Total IFN was assayed for anti-viral activity on microtitre plate cultures of human amnion WISH cells by using a 50% Sindbis virus yield reduction assay (Langford, Stanton & Johnson, 1978; Langford *et al.*, 1981). IFN-γ was determined by pre-incubation of samples at pH 3·0 and by treatment with an antibody specific for only IFN-γ.

Statistical methods. Student's *t*-test and Student's *t*-test for pairs were used to determine the statistical significance.

RESULTS

Decreased IL-2 production in PB, SF and ST of RA patients

PHA stimulated lymphocytes from the SF ($n = 15$) and PB ($n = 15$) of patients with RA (Fig. 1) produced lower quantities of IL-2 than normal PB: $28 \pm 4{\cdot}5$ (s.e.) u/ml for normal PB, $9{\cdot}3 \pm 1{\cdot}7$ for RA PB ($P < 0{\cdot}001$) and $13{\cdot}4 \pm 3{\cdot}7$ for RA SF ($P < 0{\cdot}02$). Of these, matched PB and SF were available from 13 of the patients ($14{\cdot}8 \pm 7{\cdot}3$ u/ml in SF and $10{\cdot}2 \pm 3{\cdot}3$ in matched PB). No significant difference was observed between RA SF and PB. The cultured ST cells from the four RA patients studied produced very little IL-2, $2{\cdot}6 \pm 1{\cdot}7$ (s.e.) u/ml. Lymphocytes from the SF of four patients with gout produced abundant quantities of IL-2 following PHA stimulation, $61{\cdot}5 \pm 16{\cdot}4$ u/ml. Unstimulated lymphocytes from patients with RA and gout and normal controls did not spontaneously produce detectable quantities of IL-2. IL-2 production by RA PB or SF did not correlate with NK activity (Combe *et al.*, 1984), therapeutic regimen or clinical activity as measured by erythrocyte sedimentation rate.

Decreased response of RA lymphocytes to exogenous IL-2

The proliferative response to exogenous IL-2 of activated lymphocytes from normal controls was compared to the PB and SF of patients with RA. Lymphocytes were activated by incubation of MNC with Con A. Activated cells from RA PB and SF exhibited a significantly reduced ($P < 0{\cdot}05$–$0{\cdot}001$) proliferative response to exogenous IL-2 compared to normal PB lymphocytes

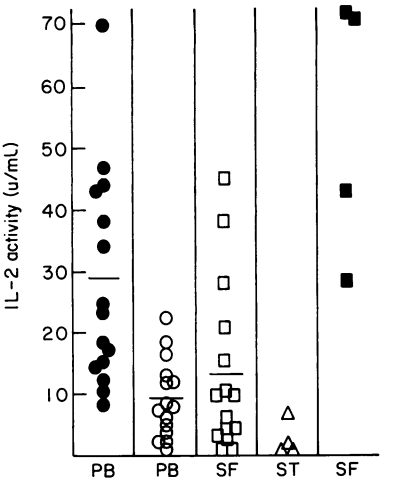

Fig. 1. IL-2 released by PHA stimulated MNC from peripheral blood (PB,○) synovial fluid (SF□) and synovial tissue (ST,△) of patients with RA, the PB of normal controls (●) and the SF of patients with gout. (■) The horizontal lines represent the means for each group.

(Table 1). The proliferative response to exogenous IL-2 of SF lymphocytes was reduced compared to those from PB ($P < 0{\cdot}05$–$0{\cdot}001$).

Decreased IFN-γ induction after treatment of RA lymphocytes with IL-2

IL-2 is capable of induction of IFN-γ from unstimulated PB human T lymphocytes (Kasahara *et al.*, 1983). Since RA PB and SF generated reduced quantities of IL-2, eight paired RA SF and PB samples were compared to eight normal PB samples for their ability to produce IFN in response to IL-2 (Fig. 2). The IFN activity released spontaneously by either RA or normal unstimulated cells was negligible. Spontaneously released IFN was detected in only four cultures. Ten units per

Table 1. Response to exogenous IL-2 by conconavilin A activated lymphocytes from RA patients and normal controls

	Number of Subjects	^{3}H-thymidine incorporation		
		− IL-2	+ IL-2 (1:4 dilution)	+ IL-2 (1:8 dilution)
Normal PB	10	681* ±173	39,685 ±4,351	37,288 ±4,227
RA PB	6	604 ±245	27,466 ±2,632	21,402§ ±1,481
RA SF	8	561 ±132	17,408‡ ±2,369	13,600† ±2,024

RA and normal mononuclear cells were activated with Con A (10 μg/ml × 48 h), then washed with α-methyl mannoside and incubated with serial dilutions of the standard IL-2 preparation for 72 h.

* Mean ct/min ± s.e.

† Different from normal PB, $P < 0{\cdot}001$; Different from RA PB, $P < 0{\cdot}001$.

‡ Different from normal PB and RA PB, $P < 0{\cdot}05$.

§ Different from normal PB, $P < 0{\cdot}05$.

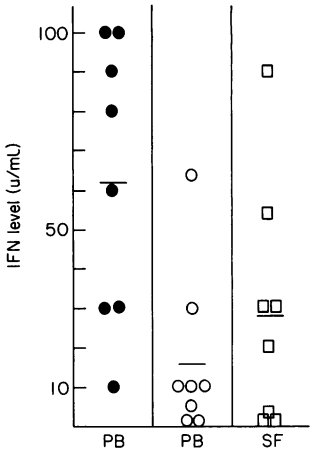

Fig. 2. IFN induced by incubation of MNG with IL-2. The groups and symbols are the same as those in Fig. 1. Spontaneously released IFN was subtracted from the individual values plotted (10 u/ml in one normal PB and two RA SF; 20 u/ml from one RA PB). The media alone, containing the exogenous IL-2, possessed 7·5 u/ml of IFN activity. This value was not subtracted from the values presented above.

millilitre was detected with one normal PB and two RA SF and 20 u/ml with one RA PB. These values were subtracted from those presented in Fig. 2. The media alone, containing exogenous IL-2, possessed 7·5 u/ml of IFN activity. This value was not subtracted from the values presented in Fig. 2 because many of the cultures had IFN values less than 7·5 u/ml suggesting absorption or utilization. With IL-2 incubation, both PB and SF from patients produced significantly less IFN than normal PB lymphocytes: $62{\cdot}5 \pm 12{\cdot}5$ (s.e.) u/ml with normal PB, $16{\cdot}1 \pm 7{\cdot}6$ u/ml with RA PB ($P < 0{\cdot}001$), and $29{\cdot}6 \pm$ u/ml with SF ($P < 0{\cdot}02$) (Fig. 2). There was no significant difference between rheumatoid SF and paired PB. The IFN produced was primarily of the γ class, since incubation at pH 3·0 and treatment with an antibody specific for γ-IFN-γ either abolished or greatly reduced the IFN activity compared to the pre-treatment values (Table 2).

Despite the defective induction of IFN by IL-2, IL-2 increased the *in vitro* NK activity of RA patients to a degree comparable to normals (Table 3). Eight subjects were available for comparison in each group. As for the larger group, IL-2 generated less IFN in RA PB and SF, although this difference did not reach statistical significance employing only these eight samples. In contrast, IL-2 augmented NK activity to a comparable (RA PB) or greater (RA SF) degree compared to normal

Table 2. Identification of IFN produced by IL-2

	Treatment		
Source	None	pH 3·0	anti-IFN-γ
Normal PB	100*	<3	3
RA PB	30	<3	6
RA SF	30	<3	3
IFN-α control	10	6	6
IFN-β control	20	10	6
IFN-γ control	10	<3	<3

* IFN activity in culture supernatants in u/ml.

PB. Thus, there was a discordance between the ability of IL-2 to stimulate IFN production and to augment NK activity in both the PB and SF of our patients with RA.

Since macrophages have been shown to suppress IFN-γ release during the autologous mixed lymphocyte response with RA PB (Hasler *et al.*, 1983a), we studied IFN production by RA SF following partial removal of macrophages by adherence to plastic. Removal of adherent cells did not restore but further decreased the IFN induced by IL-2 (Table 4). Therefore the decreased IFN production did not appear to be explained by suppressor adherent cells. These data suggest that the co-operation of adherent cells might be necessary to effect the IL-2-mediated release of IFN.

Table 3. Comparison of the effect of IL-2 on NK activity and IFN induction in patients with RA and normal controls

	Number of subjects	Increase of NK activity*	IFN titre (u/ml)†
Normal PB	8	16·2 ± 2·8 (33%)	62·5 ± 12·5
RA PB	8	13·8 ± 2·3 (47%)	16·1 ± 7·6§
RA SF	8	25·8 ± 3·0 (67%)‡	29·6 ± 11·5

* Absolute increase of NK activity expressed as percentage of total ^{51}Cr release. Mean ± s.e. at a 20:1 effector:target ratio. Value in parentheses indicates the percentage increase from baseline. The baseline values for NK activity were 48·4% cytotoxicity for normal PB, 29·3% for RA PB and 37·3% for RA SF.

† Mean ± s.e.

‡ Significantly greater than normal PB ($P < 0.05$) and RA PB ($P < 0.01$).

§ Significantly less than normal PB ($P < 0.01$).

Table 4. Effect of removal of adherent cells on IL-2 induced IFN in RA synovial fluid

	IFN titre (u/ml)*	
Expt. No.	Mononuclear cells	Nonadherent cells
1	54	0
2	20	10
3	30	0

* IFN in u/ml released by the addition of IL-2 to mononuclear cells or to non-adherent cells. Adherent cells were removed by plastic adherence.

DISCUSSION

Three aspects of IL-2 biology (production, induction of proliferation and induction of IFN-γ) were found to be diminished employing synovial and PB lymphocytes from patients with RA. Wilkins *et al.* (1983) recently reported IL-2 like activity in SF of RA patients. We did not detect spontaneous IL-2 activity in culture supernatants of unstimulated RA cells even with SF or ST where T

lymphocytes have the characteristics of activated cells (Janossy *et al.*, 1981; Klareskog *et al.*, 1982; Galili *et al.*, 1981; Burmester *et al.*, 1981). Furthermore, rheumatoid organ cultures failed to spontaneously generate IL-2, although high concentrations of interleukin-1 (IL-1) were produced (Chin *et al.*, 1984). After stimulation with PHA, RA lymphocytes from PB, SF and ST were found to produce less IL-2 compared with normal PB or SF lymphocytes from patients with gout. Additionally, activated RA cells exposed to exogenous IL-2 proliferated less well than did normal cells. This defect was greater in the synovial compartment than in PB.

There are several potential explanations for the impaired production of IL-2 by rheumatoid lymphocytes. Impaired production of IL-1 by rheumatoid monocytes seems unlikely since IL-1 activity has been reported in rheumatoid synovium by several authors who have suggested that IL-1 could play an important role in the pathogenesis of joint destruction (Mizel *et al.*, 1981; Fontana *et al.*, 1982; Wood *et al.*, 1983). Other potential mechanisms for the deficient IL-2 production in RA include inhibition by suppressor cells or by inhibitory factors, defects of binding or response to IL-1 by rheumatoid lymphocytes, or intrinsic abnormalities of the IL-2 producing T lymphocyte population. The presence of IL-2 in rheumatoid SF together with the decreased production of IL-2 *in vitro* may be explained by prior *in vitro* activation. Analysis of rheumatoid synovial cells reveals the presence of Ia positive T lymphocytes, suggesting prior activation (Burmester *et al.*, 1981; Galili *et al.*, 1981). This prior activation might be responsible for the decreased production of IL-2 *in vitro* (Silver *et al.*, 1983).

The decreased responsiveness of rheumatoid lymphocytes to exogenous IL-2 may also be explained by prior activation (Silver *et al.*, 1983) or to intrinsic T cell abnormalities. Silver *et al.* (1983) reported that the decreased proliferative response of synovial T cells to PHA could be partially restored by the addition of IL-2. Their inability to completely restore the proliferative response to PHA and the defective response of rheumatoid blasts to IL-2 noted in our study may be due to a adsorption of IL-2 by receptors on activated rheumatoid lymphocytes no longer capable of proliferation. We are currently examining rheumatoid lymphocytes with an antibody to the IL-2 receptor (anti-TAC) and a flourescence activated cell sorter to further clarify this possibility. Preliminary studies have not suggested an increase of IL-2 receptor positive cells nor an alteration of IL-2 receptor density on lymphocytes from patients with RA.

Absent IFN-γ production in cultured RA ST (Chin *et al.*, 1983) and diminished IFN-γ production in response to autologous stimulation with RA PB (Hasler *et al.*, 1983b) have been reported. We demonstrated in this study the defective production of IFN-γ in response to IL-2. Hasler *et al.* (1983b) attributed the decreased IFN-γ production generated during the autologous mixed lymphocyte response in patients with RA to the inhibitory effects of monocyte generated prostaglandins. We were unable to restore IFN-γ by removing adherent cells suggesting that excessive production of prostaglandins was not responsible for the defect observed in our study. Thus, multiple mechanisms may account for abnormalities of IFN production in patients with RA.

Despite defective IL-2-dependent IFN-γ production, treatment of rheumatoid MNC with IL-2 boosted NK activity strongly. This result suggests that IL-2 may augment NK activity in our patients through mechanisms other than the induction of IFN-γ, perhaps by directly effecting NK cells (Klein & Bevan, 1983; Handa *et al.*, 1983). These results, especially in the rheumatoid joint, suggest the diminished or inadequate production of IL-2 might be responsible for the reduced NK activity. The impaired production of IL-2 and IFN-γ *in vitro* by rheumatoid T lymphocytes supports the notion that defective T cells are involved in the pathogenesis of RA. It is possible that inadequate production of IL-2 may lead to defective T suppressor and NK activity in the rheumatoid joint. These defects could be, in part, responsible for the B cell hyperactivity with resultant immunological injury that is apparent in the rheumatoid joint.

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