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Stimulation by interleukin-7 of mononuclear cells in peripheral blood, synovial fluid and synovial tissue from patients with rheumatoid arthritis.

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Abstract

To determine how interleukin-7 (IL-7) affects the proliferation of T cells in patients with rheumatoid arthritis (RA), we evaluated the response of mononuclear cells (MNC) obtained from their peripheral blood (PB), synovial fluid (SF) and synovial tissue (ST) to stimulation by recombinant IL-7 and interleukin-2 (IL-2). Each cytokine was administered alone or combined with phytohemagglutinin (PHA). Cellular DNA synthesis was assayed by the [3H]-thymidine incorporation method. The stimulatory effect of 500 u/ml IL-7 on PBMNC obtained from 19 patients with RA was significantly lower than on PBMNC from 19 healthy controls. However, the same degree of stimulatory activity of 500 u/ml IL-2 was observed on the PBMNC from both RA patients and control subjects. The response of PBMNC to a suboptimal dose of PHA (0.2 micrograms/ml) was enhanced by adding either IL-7 or IL-2 (100 or 500 u/ml) to the cultures. The enhanced synthesis of DNA by both RA and control PBMNC on exposure to IL-7 following stimulation by a suboptimal dose of PHA was higher than that of IL-2. The effect of IL-7 on RA PBMNC was significantly greater than that of IL-2 at the concentration of 100 u/ml on PBMNC from the same RA patients. The stimulatory activity of IL-2 at the concentrations of 100 and 500 u/ml on SF MNC and ST MNC exceeded that of IL-7. In particular, an IL-2 dose of 500 u/ml had a marked effect on SF MNC. The PHA response of SF MNC was the lowest seen among the MNC from three different compartments.(ABSTRACT TRUNCATED AT 250 WORDS)

KEYWORDS: interleukin-1, interleukin-2, rheumatoid arthritis, lymphocyte proliferation, phytohemagglutinin

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Stimulation by Interleukin-7 of Mononuclear Cells in Peripheral Blood, Synovial Fluid and Synovial Tissue from Patients with Rheumatoid Arthritis

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To determine how interleukin-7 (IL-7) affects the proliferation of T cells in patients with rheumatoid arthritis (RA), we evaluated the response of mononuclear cells (MNC) obtained from their peripheral blood (PB), synovial fluid (SF) and synovial tissue (ST) to stimulation by recombinant IL-7 and interleukin-2 (IL-2). Each cytokine was administered alone or combined with phytohemagglutinin (PHA). Cellular DNA synthesis was assayed by the [³H]-thymidine incorporation method. The stimulatory effect of 500u/ml IL-7 on PBMNC obtained from 19 patients with RA was significantly lower than on PBMNC from 19 healthy controls. However, the same degree of stimulatory activity of 500u/ml IL-2 was observed on the PBMNC from both RA patients and control subjects. The response of PBMNC to a suboptimal dose of PHA (0.2μg/ml) was enhanced by adding either IL-7 or IL-2 (100 or 500u/ml) to the cultures. The enhanced synthesis of DNA by both RA and control PBMNC on exposure to IL-7 following stimulation by a suboptimal dose of PHA was higher than that of IL-2. The effect of IL-7 on RA PBMNC was significantly greater than that of IL-2 at the concentration of 100u/ml on PBMNC from the same RA patients. The stimulatory activity of IL-2 at the concentrations of 100 and 500u/ml on SF MNC and ST MNC exceeded that of IL-7. In particular, an IL-2 dose of 500u/ml had a marked effect on SF MNC. The PHA response of SF MNC was the lowest seen among the MNC from three different compartments. Thus, the stimulatory activity of IL-7 or IL-2 on RA MNC appears to differ according to the site of the body from which those cells were obtained or the state of cell activation.

Key words: interleukin-7, interleukin-2, rheumatoid arthritis, lymphocyte proliferation, phytohemagglutinin

Interleukin-7 (IL-7) was initially purified and cloned as a growth factor for murine B-cell progenitors (1-3) and human tonsillar B cells (4). Recent reports have shown that it also functions as a T-cell growth factor for both murine (5-7) and human (7-12) systems. In addition, IL-7 enhances the activity of natural killer (NK) and lymphokine-activated killer (LAK) cells (13), generates cytotoxic T lymphocytes against tumor cells (14, 15) and induces cytokine secretion by, and tumoricidal activity of, human peripheral blood monocytes (16).

The pathogenesis of rheumatoid arthritis (RA) is not defined. It is, however, known that the certain mediator molecules such as interleukin 1 (IL-1), tumor necrosis factor α (TNF α) are, at least, involved in the disease development for RA (17). The T cells which predominantly infiltrate the synovial tissue of RA patients are activated by antigens in local lesions, proliferate to give clonal expansion, and form an inflammatory exudate called the pannus (18). Interleukin 2 (IL-2) is known as a T-cell growth factor and IL-2 gene expression is increased in RA synovia (19), although there is no study about IL-7 in RA. Recent reports (8, 9) have shown that recombinant human IL-7 and IL-2 are each mitogenic on human peripheral blood mononuclear cells (PBMNC) and on T

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cell enriched (E^+) cells, and IL-7 enhances the proliferation of PBMNC stimulated by anti-CD3 monoclonal antibody or lectin via IL-2-independent mechanisms, since anti-IL-2 or anti-IL-2 receptor (Tac) monoclonal antibody did not inhibit the activity of IL-7. In addition to IL-2, this activity of IL-7 plays an important role in the physiology of T cells in humans (9). Abnormalities of T cell phenotype and function have been reported in RA patients. Such abnormalities include a decrease of suppressor/cytotoxic ($CD8^+$) T cells (20), an increase of helper/inducer ($CD4^+CD45RO^+$) T cells (21) and a low response of T cells in peripheral blood to phytohemagglutinin (PHA) (22).

These observation led us to determine how IL-7 affects the proliferation of T cells from patients with RA. In this study, we examined the effect of IL-7 and IL-2 on the synthesis of DNA in mononuclear cells from three different compartments in RA patients, *i.e.*, the peripheral blood, synovial fluid and synovial tissue, and compared them with the PBMNC obtained from control subjects.

Subjects and Methods

Patients. We studied 19 patients with RA, 5 men and 14 women, who met the diagnostic criteria of the American Rheumatism Association (ARA) (23). Their average age was 55.1 years (range 20 to 78 years). Patients were staged according to the method by Steinbrocker *et al.* (24): Stage I (1), Stage II (6), Stage III (2), and Stage IV (10). Using the functional classification, 3 patients were classified as I, 7 as II, 6 as III, and 3 as IV. Treatment administered included non-steroidal anti-inflammatory drugs (NSAIDs) (1 patient); NSAIDs plus gold sodium thiomalate (GST) (5 patients), NSAIDs plus D-penicillamine or bucillamine (7 patients), and NSAIDs plus low dose prednisolone, *i.e.*, less than 5 mg/day (6 patients). We obtained peripheral blood samples from 19 healthy subjects who were sex and age-matched to the RA patients (average 53.2 years; range 22 to 76 years) to serve as controls. In addition, to examine MNC from three different compartments in the same individuals, specimens of synovial fluid and tissue were obtained from 2 RA patients undergoing total knee replacement.

Mononuclear cell preparation. Mononuclear cells (MNC) were isolated from heparinized peripheral blood or synovial fluid with 10 u/ml of heparin (Upjohn, Tokyo, Japan) and 20 u/ml hyaluronidase (Mochida Pharmaceutical Co., Tokyo, Japan) as the interface cell layer following Ficoll (Pharmacia, Uppsala, Sweden)-Conray (Daiichi Pharmaceutical Co., Tokyo, Japan) gradient sedimentation. MNC were washed 3 times with RPMI 1640 medium

(GIBCO Lab., Life Technologies, Inc., Grand Island, NY, USA) supplemented with 100 u/ml of penicillin G, 100 μ g/ml of streptomycin and 20 mM of Hepes buffer (GIBCO). MNC were resuspended at a cell concentration of 1×10^6 /ml in RPMI 1640 medium containing 10 % of fetal bovine serum (FBS, heat-inactivated, GIBCO). MNC that infiltrated the synovial tissue of RA patients were isolated by the method of Abrahamsen *et al.* (25). Synovial tissue was obtained from 4 RA patients during total knee replacement, and teased with scissors in Ca^{++} - and Mg^{++} -free Hanks' balanced salt solution (HBSS, GIBCO). After treatment with DNase (25 μ g/ml, Sigma Chemical Co., St. Louis, MO, USA) and collagenase (200 u/ml, Worthington Biomedical Co., Freehold, NJ, USA) for 1 h at 37°C, the tissue was passed through a layer of iron mesh (pore size 210 μ m; Ikemoto, Tokyo, Japan). The cells were washed 3 times with RPMI 1640 medium and finally resuspended in Dulbecco's modified Eagle medium (GIBCO) containing 10 % FBS at a cell concentration of 1×10^6 /ml. Cells were incubated overnight at 37°C in 5 % CO_2 . The nonadherent cells (> 90 % of cells were MNC) were removed with prewarmed medium, washed 3 times with medium, and resuspended in RPMI 1640 medium with 10 % FBS at 1×10^6 /ml.

DNA synthesis by MNC. The synthesis of DNA by MNC was measured by 3H -thymidine incorporation. MNC at a concentration of 1×10^5 /well in microtiter plates (Falcon No. 3072) were cultured in RPMI 1640 medium with 10 % FBS for 72 h in 5 % CO_2 in an incubator. Sixteen h before the end of culture, 1 μ Ci/well of 3H -thymidine (Amersham, UK) was pulsed. The cells were harvested on a glass fiber filter (Labo-Science Co., Tokyo, Japan) by an automated cell harvester (Labo-Science Co.). Radioactivity was measured with a liquid scintillation counter (Aloka, Tokyo, Japan).

Reagents. Human recombinant IL-7 was purchased from Genzyme Co., Boston, Mass, USA. The specific activity of IL-7 was 1×10^7 units/mg protein as measured by bioassay for induction of 3H -thymidine uptake by mouse C3H/HeJ thymocytes. Human recombinant IL-2 was supplied by Shionogi Pharmaceutical Co., Osaka, Japan. The specific activity of IL-2 was 1×10^7 J.R.U./mg protein. Phytohemagglutinin (PHA)-P was purchased from DIFCO Laboratories, Detroit, Michigan, USA.

Statistical analysis. Data are reported as mean \pm SEM. The difference in 3H -thymidine uptake by MNC with stimulation by the cytokines or PHA and without stimulation was analyzed by Student's *t*-test. A level of $p < 0.05$ was accepted as statistically significant.

Results

Stimulatory activity of IL-7 and IL-2 on DNA synthesis of control PBMNC. Our pilot experiment confirmed the results of other investigators (8, 9), that human recombinant IL-7 as well as IL-2 stimulated the spontaneous synthesis of DNA by control PBMNC

Table 1 Stimulatory activity of human recombinant interleukin-7 (IL-7) and IL-2 on DNA synthesis of peripheral blood mononuclear cells from healthy controls after 3 days' culture.

Concentration of cytokine (u/ml)	IL-7			IL-2		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
0	651 ± 96	590 ± 104	357 ± 48	1244 ± 69	885 ± 171	533 ± 58
1	623 ± 102	637 ± 47	n. d.	2020 ± 62 ^c	1248 ± 325 ^d	n. d.
10	701 ± 93	690 ± 105	n. d.	3292 ± 161 ^d	2911 ± 114 ^d	n. d.
100	2662 ± 280 ^c	895 ± 14 ^b	730 ± 210 ^b	7128 ± 162 ^d	5792 ± 266 ^d	2990 ± 78 ^d
500	3222 ± 271 ^d	n. d.	1384 ± 136 ^a	8710 ± 609 ^d	n. d.	7886 ± 776 ^d
1000	3786 ± 60 ^d	n. d.	1753 ± 143 ^d	11055 ± 504 ^d	n. d.	8286 ± 672 ^d

Each value represents mean (± SEM) count per min of triplicate cultures. When results were compared between without and with cytokines in each group, the *p* values were less than 0.05 (a), or less than 0.02 (b), or less than 0.01 (c), or less than 0.001 (d), respectively.; Exp.: experiment number; n.d.: not done

following culture for 3 days (Table 1). The addition of IL-7 to the culture at concentrations of 10², 5 × 10² and 10³ u/ml significantly enhanced the synthesis of DNA by PBMNC. The stimulatory activity of IL-2 on PBMNC after 3 days of culture was greater than that of IL-7 (Table 1). The activity of IL-7 on control PBMNC did not exceed that of IL-2, even after culture for 14 days (data not shown).

Response of PBMNC from RA patients to IL-7. The cpm value for the spontaneous DNA synthesis of control PBMNC after 3 days' culture was 368 ± 26 (Mean ± SEM). The synthesis of DNA by control PBMNC rose when exposed to a concentration of 100 and 500 u/ml of IL-7 (668 ± 110 cpm for 100 u/ml, *p* < 0.01 and 1091 ± 135 cpm for 500 u/ml, *p* < 0.01), or of IL-2 (1693 ± 182 cpm, *p* < 0.01, 4975 ± 511 cpm, *p* < 0.01). The stimulatory activity of IL-2 at a concentration of 500 u/ml was about 5 times as high as the value of cpm obtained with IL-7. The response of PBMNC to 500 u/ml of IL-7 (729 ± 104 cpm, n = 19) was lower than that of control PBMNC (1091 ± 135 cpm, *p* < 0.05), although PBMNC from both RA and control groups responded equally to IL-2 (Fig. 1).

Response of PBMNC from RA patients to IL-7 and suboptimal dose of PHA. In a preliminary experiment, the suboptimal dose of PHA required to stimulate DNA synthesis in control PBMNC was determined as 0.2 μg/ml in the final concentration for cultures (data not shown). The addition of 100 or 500 u/ml of either IL-7 or IL-2 to the culture of PBMNC enhanced the synthesis of DNA by PBMNC that had been stimulated by a suboptimal dose of PHA (*p* < 0.01, except *p* < 0.05 for 100 u/ml of IL-2 on RA PBMNC, Fig. 2). The response of RA PBMNC stimulated by the suboptimal dose of PHA to

100 u/ml of IL-7 (35248 ± 4957 cpm, n = 19) exceeded that obtained with the same concentration of IL-2 (28455 ± 3685 cpm, *p* < 0.05) and tended to be greater

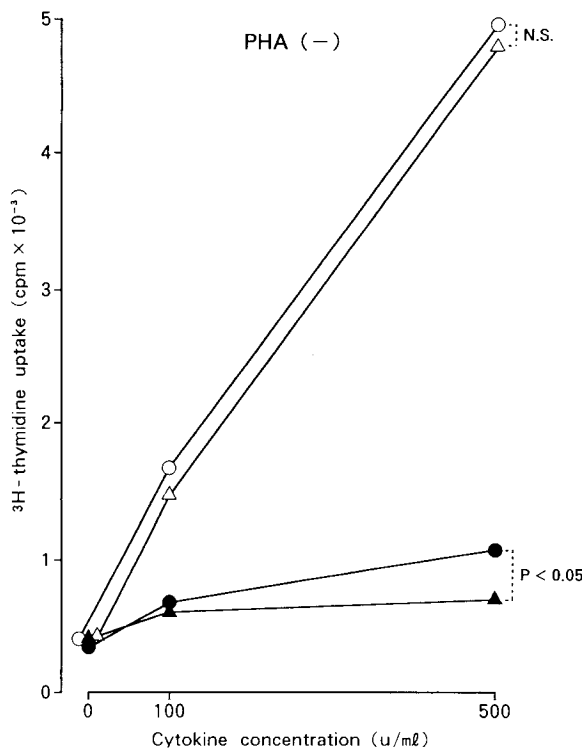


Fig. 1 Stimulatory activity of human recombinant interleukin-7 (IL-7) and interleukin-2 (IL-2) given alone on DNA synthesis of peripheral blood mononuclear cells (PBMNC) from 19 rheumatoid arthritis (RA) patients and 19 sex-age matched controls. Each point represents the mean count per min (cpm) value of 19 individuals. (○): effect of IL-2 on control PBMNC, (△): effect of IL-2 on RA PBMNC, (●): effect of IL-7 on control PBMNC, (▲): effect of IL-7 on RA PBMNC, *p* < 0.05 (RA vs control), N. S.: not significant

than that of control PBMNC to 100 u/ml of IL-7 (32372 ± 3113 cpm, $n = 19$, Fig. 2). RA and control PBMNC showed a similar value of ^3H -thymidine uptake when 500 u/ml of IL-7 or of IL-2 was added to the PBMNC culture that had been stimulated by a suboptimal dose of PHA (Fig. 2).

Response of RA mononuclear cells in synovial fluid and tissue to IL-7. Mononuclear cells from the synovial fluid (SF MNC) obtained from 6 RA patients were stimulated by either 100 or 500 u/ml of IL-2 ($p < 0.05$, Fig. 3) and 500 u/ml of IL-7 ($p < 0.05$, Fig. 3). The response of SF MNC to 500 u/ml of IL-2 (12848 ± 3395 cpm, $n = 6$) was higher than that obtained with the same

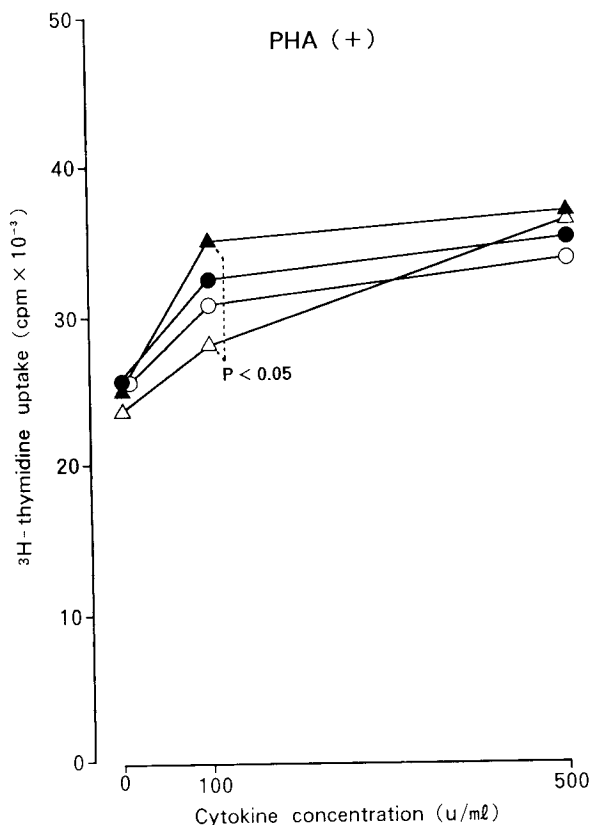


Fig. 2 Augmented activity of human recombinant interleukin-7 (IL-7) and interleukin-2 (IL-2) on synthesis of DNA by peripheral blood mononuclear cells (PBMNC) from 19 rheumatoid arthritis (RA) patients and 19 sex-age matched controls in response to stimulation by a suboptimal dose of phytohemagglutinin (PHA). Each point represents the mean count per min (cpm) value of 19 individuals. (○): effect of IL-2 on control PBMNC, (△): effect of IL-2 on RA PBMNC, (●): effect of IL-7 on control PBMNC, (▲): effect of IL-7 on RA PBMNC, $p < 0.05$ (IL-7 vs IL-2 at the concentration of 100 u/ml on RA PBMNC)

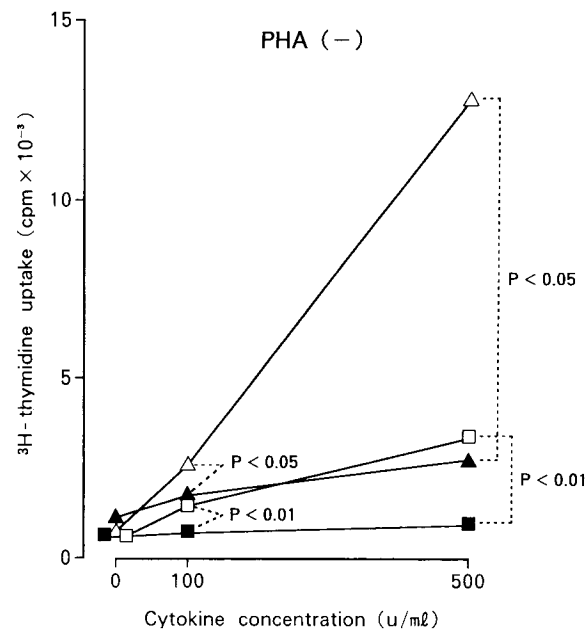


Fig. 3 Stimulatory activity of human recombinant interleukin-7 (IL-7) and interleukin-2 (IL-2) on synthesis of DNA by mononuclear cells (MNC) in synovial fluid (SF) from 6 rheumatoid arthritis (RA) patients and in synovial tissue (ST) from 4 RA patients. Each point represents the mean count per min (cpm) of 4 or 6 experiments. (△): effect of IL-2 on SF MNC, (▲): effect of IL-7 on SF MNC, (□): effect of IL-2 on ST MNC, (■): effect of IL-7 on ST MNC, $p < 0.05$ (IL-2 vs IL-7 at the concentration of 500 u/ml on SF MNC, $p < 0.01$ (IL-2 vs IL-7 at the concentration of 500 u/ml on ST MNC), $p < 0.05$ (IL-2 vs IL-7 at the concentration of 100 u/ml on SF MNC), $p < 0.01$ (IL-2 vs IL-7 at the concentration of 100 u/ml on ST MNC)

concentration of IL-7 (2672 ± 849 cpm, $n = 6$, $p < 0.05$, Fig. 3). Mononuclear cells from the synovial tissue (ST MNC) obtained from 4 RA patients were also stimulated by 100 or 500 u/ml of IL-2 alone (1424 ± 123 cpm, 3349 ± 601 cpm, $p < 0.05$, respectively), compared with the spontaneous synthesis of DNA (533 ± 99 cpm) after 3 days' culture. However, IL-7 (618 ± 119 cpm for 100 u/ml, 892 ± 182 cpm for 500 u/ml, respectively) had no significant effect on ST MNC. The effect of IL-2 (500 u/ml) on ST MNC was stronger than that of IL-7 ($p < 0.01$, Fig. 3).

Response of RA MNC in synovial fluid and tissue to IL-7 and suboptimal dose of PHA. The response of SF MNC (7232 ± 1598 cpm, $n = 6$) to a suboptimal dose of PHA was lower than that of ST MNC (13060 ± 2423 cpm, $n = 4$) and RA PBMNC (25531 ± 3348 cpm, $n = 19$). However, the co-addition of IL-7 or IL-2 with PHA to the cultures further enhanced DNA synthesis of SF

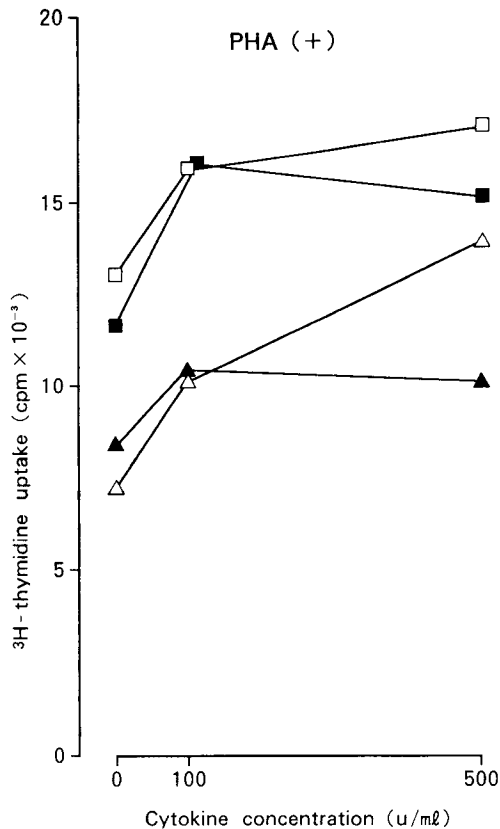


Fig. 4 Augmented activity of human recombinant interleukin-7 (IL-7) and interleukin-2 (IL-2) on synthesis of DNA by mononuclear cells (MNC) in synovial fluid (SF) from 6 rheumatoid arthritis (RA) patients and in synovial tissue (ST) from 4 RA patients during stimulation by suboptimal dose of phytohemagglutinin (PHA). Each point represents the mean count per min (cpm) of 4 experiments. (△): effect of IL-2 on SF MNC, (▲): effect of IL-7 on SF MNC, (□): effect of IL-2 on ST MNC, (■): effect of IL-7 on ST MNC

MNC and ST MNC. IL-2 at concentrations of 100 and 500 u/ml exhibited a dose-dependent activity on SF and ST MNC. In contrast, the activity of 100 u/ml of IL-7 on both SF and ST MNC exceeded that of 500 u/ml of IL-7 (Fig. 4). The activity of 500 u/ml of IL-2 on both SF and ST MNC exceeded that of the same concentration of IL-7 (Fig. 4).

Discussion

Resting T cells of humans are stimulated by specific antigens which bind to T cell receptor molecules, and proliferate more effectively on exposure to T cell growth factors including IL-1, IL-2, IL-4, TNF, IL-6 and

GM-CSF (26). Other investigators have recognized that IL-7 provides strong co-stimulatory signal for the activation and proliferation of T cells by anti-CD3 monoclonal antibody or lectins, and that IL-7 has a mitogenic effect on human lymphocytes (8, 9). We also confirmed that IL-7 as well as IL-2 has a mitogenic effect on PBMNC from healthy subjects (Table 1). However, the stimuli for human PBMNC may be present in heterologous serum, *i.e.*, fetal bovine serum contained in culture medium, and/or cells that express HLA-DR antigens that induce autologous mixed lymphocyte cultures (27). Thus, these cytokines may act as T cell growth factors rather than mitogens.

PBMNC included different leukocyte subsets such as T, B, NK cells and monocytes. In order to know on which subsets IL-7 acts, we separated control PBMNC into E-rosette forming cells (E-RFC, T cells) and non-E-RFC. We observed that T cells were the ones that responded to IL-7 (data not shown), which is consistent with the findings of Londei *et al.* (8).

At a concentration of 500 u/ml, the stimulatory activity of IL-7 on RA PBMNC was significantly lower than that of control PBMNC (Fig. 1). There was, however, no difference in the activity of IL-2 between RA and control PBMNC (Fig. 1). The degree of low responsiveness of RA PBMNC to IL-7 was correlated neither with the value of CRP as a marker for joint inflammation nor with disease stage (data not shown). In contrast, the co-stimulatory activity of IL-7 given with a suboptimal dose of PHA on RA PBMNC tended to be higher than on control PBMNC. In addition, this activity of IL-7 on RA PBMNC was significantly greater than that of IL-2 at a concentration of 100 u/ml (Fig. 2). Thus, RA PBMNC showed a low response when stimulated with IL-7 alone and a high response when stimulated with IL-7 plus a suboptimal dose of PHA. Although the reason for this low or high responsiveness of RA PBMNC to IL-7 is unknown, there might be an abnormal proportion of T-cell subsets responding to IL-7 or a natural aberration of the T-cell activation pathway. Welch *et al.* (7) reported that CD4⁺ and CD8⁺ T cells were equally stimulated by IL-7, and that memory T cells (CD45R⁻) responded better to IL-7 than unprimed T cells (CD45R⁺). Emery *et al.* (21) reported an increased percentage of memory T cells in peripheral blood of RA patients. Considering those findings, one would expect that the responsiveness of RA PBMNC to IL-7 would be high. However, the biphasic response of RA PBMNC to IL-7 that was

demonstrated in this study could not be explained by the abnormal percentage of T cell subsets in RA patients.

Combe *et al.* (28) previously reported that the concanavalin-A (Con-A)-stimulated lymphoblasts from RA PBMNC responded defectively to exogenous IL-2. In our study, the responsiveness of RA PBMNC to IL-2 did not differ from that of control PBMNC either with or without the stimulation of a suboptimal dose of PHA (Figs. 1, 2). This difference may be because PHA differs from Con-A with respect to the T-cell populations stimulated by PHA and the profile of cytokines produced, and because IL-2 and PHA were simultaneously added from the beginning of the culture in our study.

In contrast to the responsiveness of PBMNC to the cytokines, the responsiveness of SF MNC to IL-2 was significantly higher than that of IL-7 or of ST MNC to both cytokines (Fig. 3). These results could be explained by the report of Lemm *et al.* (29), who found that the expression of the IL-2 receptor was enhanced by SF MNC. *In vivo* activation of SF MNC occurred, and those cells used IL-2 rather than IL-7 for their proliferation *in vitro*.

Mononuclear cells from RA synovial fluid (SF MNC) showed a lower to PHA stimulation than either the PBMNC or ST MNC (Fig. 4), consistent with the findings of Corrigan *et al.* (30). The co-stimulatory activity of IL-7 on SF and ST MNC with suboptimal dose PHA was equivalent to that of IL-2 at the concentration of 100 u/ml (Fig. 4). It is possible that SF and ST MNC from RA patients had already been activated *in vivo*, while PBMNC were resting. The difference between PBMNC and SF or ST MNC in their responsiveness to IL-7 or IL-2 with or without PHA was clearly shown when MNC from the three different compartments was obtained from the same individual (data not shown). The differing responses to IL-7 and IL-2 by MNC among the three different compartments may depend on the stage of *in vivo* cell activation.

The marked responsiveness of RA PBMNC to IL-7 following stimulation by a suboptimal dose of PHA suggests the existence of an abnormal T cell function in patients with RA, leading to an augmented immune response in the IL-7-dependent growth pathway when the T cells migrate to and are activated by antigens at the site of chronic joint inflammation.

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