Acta Medica Okayama

Volume 44, *Issue* 6 1990 *Article* 4

DECEMBER 1990

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Abstract

Twenty-four patients with rheumatoid arthritis (RA) and 20 normal controls were examined for the ability of their peripheral blood B cells to produce interleukin 1 (IL-1) with or without lipopolysaccharide (LPS). B cells were purified from peripheral blood by negative selection methods (i.e., removal of adherent cells and sheep red blood cell rosette-forming cells, followed by treatment with monoclonal antibodies (OKT3 and OKM1) and complement). The amount of IL-1 in B cell culture supernatants (SN) was measured by thymocyte and fibroblast proliferation assays and an enzyme-linked immunosorbent assay for IL-1 alpha and beta. As a group, cultured B cells from patients with RA, both spontaneously and when stimulated with LPS, produced higher levels of IL-1 than those from normal controls. IL-1 production by RA B cells with LPS had a weak but positive correlation with disease activity. Moreover, RA B cell culture SN with elevated levels of IL-1 had a synergistic effect on the growth of anti-human IgM (anti-mu) stimulated B cells. In separate experiments, the growth of RA B cells was significantly promoted by IL-1 beta both with and without anti-mu stimulation. These results suggest that B cell-derived IL-1 may be involved in the B cell clonal expansion of RA through its own activity as a B cell stimulatory factor.

KEYWORDS: rheumatoid arthritis, B cells, interleukin 1, B cell stimulatory factor

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*PMID: 2075829 [PubMed - indexed for MEDLINE]

Acta Med Okayama 44 (6) 301-308 (1990)

Increased Ability of Peripheral Blood B Cells from Patients with Rheumatoid Arthritis to Produce Interleukin 1 *In Vitro*

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Twenty-four patients with rheumatoid arthritis (RA) and 20 normal controls were examined for the ability of their peripheral blood B cells to produce interleukin 1 (IL-1) with or without lipopolysaccharide (LPS). B cells were purified from peripheral blood by negative selection methods (*i.e.*, removal of adherent cells and sheep red blood cell rosette-forming cells, followed by treatment with monoclonal antibodies (OKT3 and OKM1) and complement). The amount of IL-1 in B cell culture supernatants (SN) was measured by thymocyte and fibroblast proliferation assays and an enzyme-linked immunosorbent assay for IL-1 α and β. As a group, cultured B cells from patients with RA, both spontaneously and when stimulated with LPS, produced higher levels of IL-1 than those from normal controls. IL-1 production by RA B cells with LPS had a weak but positive correlation with disease activity. Moreover, RA B cell culture SN with elevated levels of IL-1 had a synergistic effect on the growth of anti-human IgM (anti-μ) stimulated B cells. In separate experiments, the growth of RA B cells was significantly promoted by IL-1β both with and without anti-μ stimulation. These results suggest that B cell-derived IL-1 may be involved in the B cell clonal expansion of RA through its own activity as a B cell stimulatory factor.

Key words : **rheumatoid arthritis, B cells, interleukin 1, B cell stimulatory factor**

Rheumatoid arthritis (RA) is a chronic inflammatory disease which mainly affects synovial tissue and involves an aberrant immunological process. RA is also often associated with hypergammaglobulinemia and rheumatoid factor (RF) in serum, and immunologically activated B cells have been shown to infiltrate synovial tissue to varying degrees (1). There are a number of reports on B cell abnormalities in the circulation of RA. Most notably these include an increase in the ratio of surface immunoglobulin (sIgM) to sIgD (2), enhanced spontaneous RF production *in vitro* (3), an elevated frequency of Epstein-Barr virus (EBV)-infected B cells (4), and increased numbers of CD5-positive B cells (5). These findings imply an important role of B cells in the pathogenesis of RA.

Interleukin 1 (IL-1) has a wide spectrum of immunoregulatory and inflammatory activities and is produced by different cell types, although it was originally designated as a lymphocyte activating factor (LAF) produced by the monocyte-macrophage lineage (6). It has recently been demonstrated that B cells are also efficient IL-1

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producers (7, 8). Previous studies have indicated that IL-1 is involved in the clonal expansion and differentiation of B cells (9-14), although its exact role remains unclear. It is, therefore, possible that B cell-derived IL-1 can function as an autoregulatory factor.

We studied the ability of peripheral blood B cells from patients with RA to produce IL-1 with or without lipopolysaccharide (LPS) using two routine bioassay methods, thymocyte and fibroblast proliferation assays, and an enzyme-linked immunosorbent assay (ELISA) for IL-1α and β. Our results showed increased IL-1 production *in vitro* by RA B cells, and indicated that B cell-derived IL-1 may contribute to the B cell expansion characteristic of RA through its B cell stimulatory factor (BSF) activity.

Materials and Methods

Subjects. Twenty-four patients with classical or definite RA (3 males and 21 females) as defined by the American Rheumatism Association criteria (15) were studied. The mean age was 49 (range 15-76) years, and the mean duration of disease was 5.3 years (range 0.4-16.0). Twenty patients were receiving nonsteroidal anti-inflammatory drugs, 12 prednisolone ($<$ 10 mg/day), 7 gold therapy, and 4 D-penicillamine. Normal controls consisted of 20 healthy subjects (3 males and 17 females) with a mean age of 44 (range 27-79) years.

Materials. For the IL-1 bioassay, 4 to 8-week-old C3H/HeJ mice were sacrificed to obtain thymus tissue, and normal human skin fibroblasts (CRL 1445) were obtained from the American Type Culture Collection, Rockville, MD, USA. Plastic dishes, culture plates and microtiter plates for cell culture were purchased from Becton Dickinson Labware, Lincoln Park, NJ, USA, and microtiter plates for the ELISA from Dynatech Labs., Inc., Chantilly, VA, USA. The following reagents were purchased: OKT3 and OKM1 from Ortho Diagnostic Systems Inc., Raritan, NJ, USA; B1 from Coulter Immunology, Hialeah, F1, USA; RPMI 1640 medium, Hepes buffer, Dulbecco's minimum essential medium (DMEM) and trypsin from GIBCO Labs., Grand Island, NY, USA; 2-aminoethylisothiouronium bromide (AET) and phytohemagglutinin (PHA) from Sigma Chemical Co., St. Louis, MO, USA; rabbit complement (Low-Tox-H) from Cedarlane Lab., Hornby, Ontario, Canada; Lipopolysaccharide (LPS; E. Coli 055: B5) from Difco Labs., Detroit, MI, USA; F $(ab')_2$ fragment of goat anti-human IgM (anti-μ) from Cooper Biomedical Inc., Malvern, PA, USA; *Staphylococcus aureus* Cowan strain I (SAC) from Calbiochem Corp., La Jolla, CA, USA, and [^{3}H] thymidine (^{3}H-TdR; 2 Ci/mmol) from Amersham Plc, UK. Recombinant human IL-1α and β (rIL-1α/β), monoclonal anti-human IL-1α and β antibody (monoclonal anti-IL-1α/β), and polyclonal rabbit anti-human IL-1α and β antiserum (polyclonal anti-IL-1α/β) were kindly provided by Dr. Y. Hirai (Otsuka Pharmaceutical Co., Tokushima, Japan).

Preparation of peripheral blood B cells. Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood samples by centrifugation over Ficoll-Hypaque gradients. A B cell-enriched fraction was obtained from PBMC by removal of adherent cells after a 60 min-incubation in fetal calf serum (FCS; heat-inactivated)-coated plastic dishes (Falcon #3003) at 37°C (16) and rosette-forming cells with AET-treated sheep red blood cells (17). These cells were further treated with a mixture of monoclonal antibodies to CD3 and CD11b (OKT3 and OKM1) and rabbit complement. The resulting cell preparations were more than 90% sIg-and CD20-(B1) positive, and less than 3% nonspecific esterase staining.

Culture conditions for B cells. One million B cells were cultured in 1 ml complete medium, RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), 25 mM Hepes and 10% FCS, in 24-well tissue culture plates (Falcon # 3047) at 37°C in a humidified 5% CO_2 atmosphere with or without 1 or 20 μg/ml LPS. After 24 or 48 h, culture supernatants (SN) were collected, filter sterilized, and stored at -20°C until assayed.

Assay for IL-1 (18)

Thymocyte proliferation assay. Single-cell thymocyte suspensions from C3H/HeJ mice, at 1.5×10^6 cells/200 μl/well in complete medium with 5×10^{-5} M 2-mercaptoethanol (2ME) and 2 μg/ml PHA, were cultured for 72 h with serially diluted test samples. Cultures were pulsed with 0.5 μCi ^{3}H-TdR per well for 16 h and then harvested onto glass-fiber filter. The radioactivity was determined in a liquid scintilation counter. IL-1 activity was quantified by the probit analysis (19). rIL-1β, used as the standard, was arbitrarily assigned to have 2×10^7 U of LAF activity per 1 mg protein.

Fibroblast proliferation assay. Normal human

skin fibroblasts, at 1×10^4 cells/100 μl/well in DMEM with 10 % FCS and 10^{-5} M indomethacin, were allowed to adhere overnight and cultured further for 72 h with serially diluted test samples. After a 16-h pulse of 0.5 μCi ^{3}H-TdR, fibroblasts were detached from the wells with trypsin (0.5 mg/ml) and ethylenediaminetetraacetic acid (EDTA) (0.5 mM) in phosphate buffered saline (PBS) and then harvested. IL-1 activity was determined as in the thymocyte assay.

ELISA for IL-1α and β. Microtiter plates were coated with monoclonal anti-IL-1α/β overnight at 4 °C, blocked with 0.1 % skim milk in PBS, and test samples and standards (rIL-1α/β) were added to each well. After an overnight-incubation at 4 °C, plates were washed with 0.05 % Tween in PBS. Polyclonal anti-IL-1α/β was added, the plates were incubated for 2 h at 37 °C. After washing, peroxidase-conjugated anti-rabbit Ig was added and incubated for 2 h at 37 °C. Finally, plates were washed, substrate solution (0.25 mg/ml o-phenylenediamine and 0.05 % H_2O_2 in citrate phosphate buffer) was added, and after 5 to 15 min optical density at 492 nm was read on an automatic ELISA reader. The concentration of IL-1 was expressed in ng/ml calculated from the standard curve.

Assay for B cell growth factor (BCGF) activity. Normal peripheral B cells were purified as described above. These B cells, at 2×10^5 cells/200 μl/well in complete medium with 5×10^{-5} M 2ME and 25 μg/ml anti-μ, were cultured for 72 h with 1 : 4 diluted test samples. The incorporated radioactivity was determined after a 16-h pulse of 0.5 μCi ^{3}H-TdR.

Assay for B cell differentiation factor (BCDF) activity. Purified normal B cells were stimulated in a 3-day culture with 0.01 % SAC at 1×10^6 cells/ml in complete medium. After washing with RPMI 1640, these B cells were recultured at a density of 1×10^6 cells/ml for 6 days with 1 : 3 diluted test samples. Ig production in each culture SN was measured using an ELISA.

Statistical methods. Results were analyzed by Student's *t*-test.

Results

Increased IL-1 production by B cells from patients with RA. IL-1 production by B cells cultured for 48 h with or without 1 or 20 μg/ml LPS was measured by the thymocyte assay (Fig. 1). B cells produced only small amounts of IL-1 without any stimulation and large amounts of IL-1 after LPS stimulation in a dose dependent manner. On a per-cell basis, IL-1 production by B cells with LPS stimulation averaged about two-thirds of autologous monocytes, but in some patients with RA, the levels of B cells were even higher than those of monocytes (Data not shown). The mean IL-1 production by RA B cells with either 1 or 20 μg/ml LPS was significantly higher than that of normal controls.

As individual variation was noted, patients with RA were divided into two groups based on the levels of IL-1 production by B cells with 20 μg/ml LPS, those with lower IL-1 production ($<$ 229 U/ml, which is the value of the mean plus SD of normal controls; n=13) (group A) and those with higher production ($>$ 229 U/ml; n=11) (group B). The disease activity in group B was more severe than is group A as assessed by the erythrocyte sedimentation test (A *vs* B;

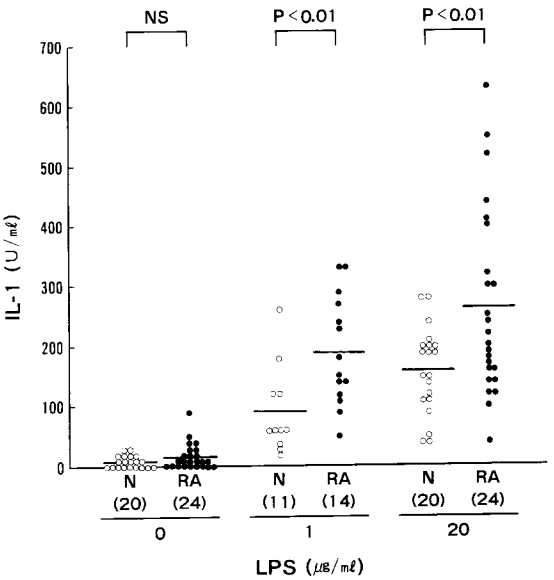

Fig. 1 Interleukin 1 (IL-1) production by B cells from patients with rheumatoid arthritis (RA) and normal controls (N). B cells were cultured for 48 h with or without lipopolysaccharide (LPS) (1 or 20 μg/ml), and the amount of IL-1 in B cell culture supernatants was measured by the thymocyte proliferation assay. Horizontal lines indicate the mean. The numbers of subjects are shown in parentheses. NS, not significant.

Table 1 Comparison of interleukin 1 (IL-1) produced by B cells from patients with rheumatoid arthritis (RA) and normal controls as measured by three different assays[a]

Assay	Interleukin 1			
	Without LPS		With LPS (20 μg/ml)	
	Control (n = 20)	RA (n = 24)	Control (n = 20)	RA (n = 24)
Thymocyte proliferation assay (U/ml)	10 ± 2	17 ± 4	159 ± 16	263 ± 31**
Fibroblast proliferation assay (U/ml)	11.6 ± 2.0	21.2 ± 3.6*	186.9 ± 16.1	303 ± 32.2**
ELISA for IL-1α (ng/ml)	0.07 ± 0.01	0.10 ± 0.01	0.59 ± 0.06	1.06 ± 0.13**
ELISA for IL-1β (ng/ml)	1.0 ± 0.1	1.7 ± 0.3*	11.1 ± 1.1	16.9 ± 1.5**

a: The amount IL-1 in 48-h B cells culture supernatants with or without 20 μg/ml lipopolysaccharide (LPS) was measured by the thymocyte or fibroblast proliferation assay and ELISA. rIL-1β used as the standard in the bioassay, was arbitrarily defined to have 2×10^7 U of biological activity per 1 mg protein. Results are expressed as the mean ± SE.
*; $p < 0.05$, **; $p < 0.01$.

mean ± SE, 53 ± 13 *vs* 68 ± 14 mm/h), hemoglobin level (11.5 ± 0.5 *vs* 11.0 ± 0.6 g/dl), serum γ-globulin (1.72 ± 0.13 *vs* 1.89 ± 0.12 g/dl), C-reactive protein (3.8 ± 0.8 *vs* 6.8 ± 2.0 mg/dl), and titer of RF (2+/1+/−; 3/7/3 *vs* 4/7/0), although not significantly so.

IL-1 production by B cells in the fibroblast proliferation assay. The fibroblast assay was over 10-fold more sensitive than the thymocyte assay, and the values of IL-1 in the fibroblast assay showed a tendency to be higher than those in the thymocyte assay (Table 1). This may have been caused by fibroblast proliferation factors distinct from IL-1 or by the presence of IL-1 inhibitors specific for LAF activity (20). In any event, spontaneous IL-1 production by RA B cells was also significantly higher than that of normal controls in this bioassay.

IL-1 production by B cells in the ELISA. The immunoassay was able to measure IL-1α and β separately, even in the presence of IL-1 inhibitors and other molecules with similar biological activities. The lowest detectable value of IL-1α and β in the ELISA was approximately 0.01–0.04 ng/ml and 0.1–0.4 ng/ml, respectively. There were good correlations ($r > 0.8$) between all three assays with the largest values (IL-1α plus β) in the ELISA, and thus the results of bioassays were verified at the protein level (Table 1).

Of two different species of IL-1, the β-form was over 15-fold higher than the α-form in both

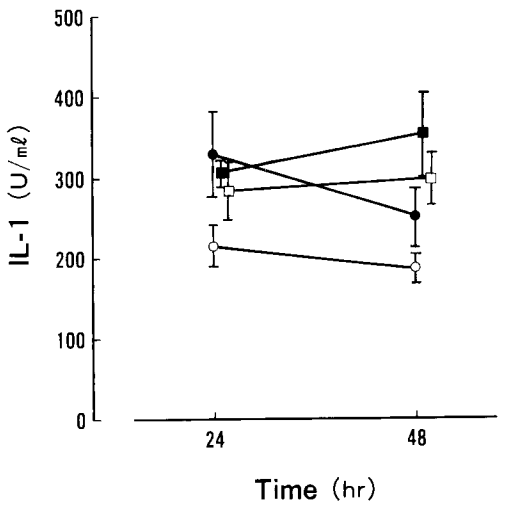

Fig. 2 Kinetics of interleukin 1 (IL-1) production by B cells (B) or monocytes (Mo) from patients with rheumatoid arthritis (RA) and normal controls (N). Autologous monocytes were prepared from adherent cells (> 85 % nonspecific esterase staining cells). B cells and monocytes were cultured for 24 or 48 h with 20 μg/ml lipopolysaccharide, and IL-1 production was measured by the thymocyte proliferation assay. Results are expressed as the mean ± SE. The numbers of subjects are shown in parentheses. (●): RA, B (n = 16); (○): N, B (n = 12); (■): RA, Mo (n = 5); (□): N, Mo (n = 3).

groups. Moreover, LAF activity in B cell culture SN was neutralized completely by a mixture of anti-IL-1α and β (Data not shown), indicating the absence of B cell-derived IL-1 molecules distinct from both forms (21, 22). Thus, the β-form was the dominant IL-1 produced in B cell culture.

Kinetics of IL-1 production by B cells. The average IL-1 production by B cells peaked in 24 h after LPS stimulation and decreased within 24 and 48 h, whereas IL-1 production by monocytes increased (Fig. 2). The mean percent reduction of patients with RA was higher than that of normal controls (24 % *vs* 14 %).

BSF activity in B cell culture SN from patients with RA. To explore the postulated activity of IL-1 as an autoregulatory factor, B cell culture SN was assayed for BCGF and BCDF activity. Forty-eight-h B cell culture SN with 20 μg/ml LPS from patients with RA, especially in group B (with higher IL-1 production), had a synergistic effect on the proliferation of anti-μ stimulated B cells, although this activity was less potent than BCGF activity produced by PHA-stimulated T cells (Fig. 3). This synergistic effect was eliminated almost completely by polyclonal anti-IL-1α and β (Data not shown). However, 24-h B cell culture SN with 20μg/ml LPS possessed no BCDF activity in either patients

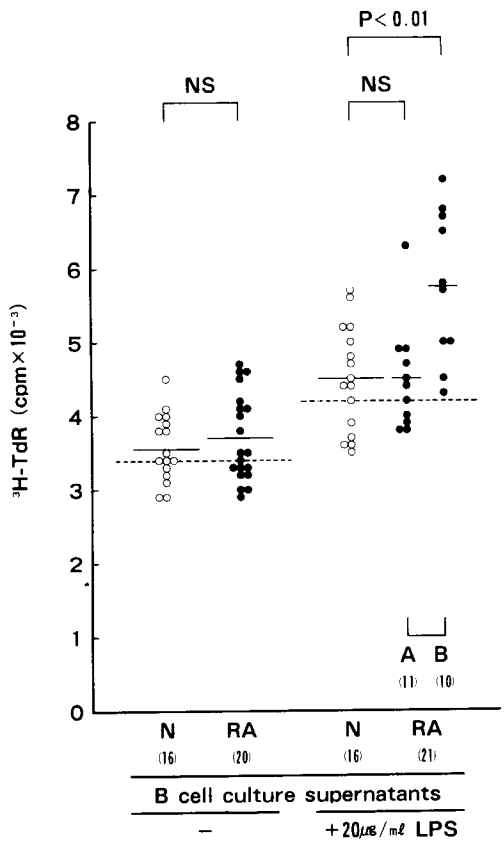

Fig. 3 B cell growth factor (BCGF) activity in B cell culture supernatants (SN) from patients with rheumatoid arthritis (RA) and normal controls (N). B cell culture (48 h) SNs with or without 20 μg/ml lipopolysaccharide (LPS) were assayed for BCGF activity as described in Materials and Methods. B cell culture SNs with LPS from RA patients were divided into two groups based on the levels of interleukin 1 in the thymocyte proliferation assay: A, < 229 U/ml and B, > 229 U/ml. The B cell proliferation by phytohemagglutinin-stimulated T cell culture SN was 14.2 ± 1.9 × 10^3 cpm (mean ± SE of 5 RA patients). Horizontal lines indicate the mean. Dotted lines indicate B cell proliferation without culture SN (with medium alone and with 20 μg/ml LPS in medium). The numbers of subjects are shown in parentheses. NS, not significant.

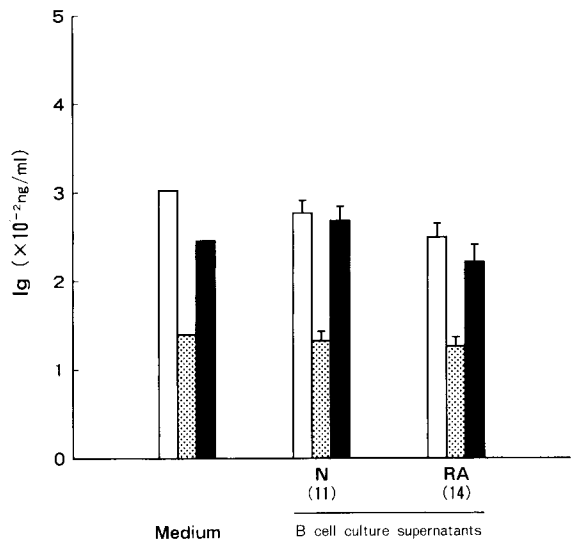

Fig. 4 B cell differentiation factor (BCDF) activity in B cell culture supernatants (SN) from patients with rheumatoid arthritis (RA) and normal controls (N). B cell cuture (24 h) SNs with 20 μg/ml LPS were assayed for BCDF activity as described in Materials and Methods. Results are expressed as the mean ± SE. The numbers of subjects are shown in parentheses. (□) IgG, (▨) IgA, (■) IgM.

Table 2 Effect of interleukin 1 (IL-1) β on the growth of B cells from patients with rheumatoid arthritis (RA) and normal controls[a]

IL-1 (U/ml)	B cell growth			
	Without anti-μ		With anti-μ (20 μg/ml)	
	Control (n = 14)	RA (n = 14)	Control (n = 14)	RA (n = 14)
0	1,150 ± 220	1,280 ± 160	2,250 ± 280	2,220 ± 260
37	1,220 ± 220 (1.11 ± 0.05	1,690 ± 220 1.35 ± 0.12)	2,110 ± 280) (0.94 ± 0.04	2,490 ± 270 1.16 ± 0.09)*
111	1,260 ± 210 (1.15 ± 0.05	1,830 ± 270 1.44 ± 0.14)	2,390 ± 320 (1.07 ± 0.06	2,760 ± 340 1.25 ± 0.09)
333	1,340 ± 260 (1.18 ± 0.06	1,890 ± 200 1.52 ± 0.10)**	2,420 ± 300 (1.09 ± 0.05	3,300 ± 440 1.58 ± 0.27)
1,000	1,380 ± 230 (1.26 ± 0.06	2,020 ± 220 1.63 ± 0.11)**	2,490 ± 330 (1.11 ± 0.03	3,350 ± 410 1.57 ± 0.15)*

a: Purified B cells, at 1×10^5 cells/220 μl in RPMI 1640 medium with 10 % FCS and 5×10^{-5} M 2-mercaptoethanol, were cultured for 72 h with or without 25 μg/ml anti-human IgM (anti-μ), with serially diluted rIL-1β (0–1,000 U/ml). [^{3}H] thymidine (^{3}H-TdR) incorporation was determined after a 16-h pulse.

Results are expressed as the mean ± SE of ^{3}H-TdR incorporation (cpm) and stimulation indices (*i.e.*, ratios of ^{3}H-TdR incorporation by B cells cultured with IL-1β to the incorporation without IL-1β) (in parentheses).

Statistical analysis: *, $p < 0.05$; **, $p < 0.01$.

with RA or normal controls (Fig. 4).

Effect of IL-1 on the B cell growth of patients with RA. In separate experiments, purified B cells from 14 patients with RA and 14 normal controls were cultured with serially diluted rIL-1β (0–1,000 U/ml) for 72 h at a density of 1×10^5 cells/200 μl in complete medium with or without anti-μ, and their proliferative response was determined by ^{3}H-TdR incorporation (Table 2). The B cell proliferation of RA was enhanced by IL-1β in a dose dependent manner even without any stimulation. The response of B cells from RA patients was significantly greater than that of B-cells from normal controls both with or without anti-μ when expressed as stimulation indices.

Discussion

Evidence that normal human B cells can produce IL-1 was originally presented by Matsushima *et al.* (7). We showed in this study that peripheral blood B cells from patients with RA, both spontaneously and with LPS stimulation, produced higher levels of IL-1 than B cells from normal controls. According to Matsushima *et al.*, the large B cell fraction, comprising *in vivo* activated B cells, produces higher levels of IL-1 in response to LPS than the small resting B cell fraction, using B cells fractionated on Percoll gradients. Thus, our results may simply represent a consequence of *in vivo* B cell activation in this disease, which has already been documented by other studies (2, 3). Moreover, IL-1 production by RA B cells had a weak but positive correlation with disease activity. Therefore, increased IL-1 production may be coincident with an active disease state of RA, in which activated B cells are increased in the circulation (2).

It is also conceivable that B cells from patients with RA represent a B cell subset(s) capable of greater IL-1 production. In fact, a number of studies have documented that a particular phenotype of B cell subsets is expanded in the circulation of RA, such as *in vivo* EBV-infected B cells (4), mouse erythrocyte rosette-forming cells (2, 23) and CD5-positive B cells (5). A recent study on EBV-transformed B cells has indicated that B cells are heterogeneous in their capacity to

produce IL-1 (23). Further studies will be necessary to identify the responsible B cell subset(s).

The IL-1 activity in culture supernatants of RA B cells but not monocytes was decreased within 24 and 48h, as shown in Fig. 2. This reduction may be due, at least in part, to the production of IL-1 inhibitors or the absorption and utilization of IL-1 by B cells. The former possibility was suggested by our finding that some RA B cell culture SN inhibited LAF activity at low dilutions. An IL-1 inhibitor derived from an EBV-transformed B cell has been reported (24), indicating the capacity of B cells to produce such factors.

RA B cell culture SN with higher levels of IL-1 promoted moderate B cell growth in synergy with anti-μ. This BCGF activity is believed to be derived from IL-1 or a closely related moiety, since it was blocked by anti-IL-1. There is some evidence that IL-1 may be required for B cell development. Anti-IL-1 inhibits the growth and differentiation of pokeweed mitogen-stimulated B cells (10).

More recently, it was shown that B cells express functional receptors for IL-1 stimulation (26). While as yet no consensus exists about the role of IL-1 in B cell development, our results correspond most closely to those of Folkoff *et al.* (11, 12).

We also showed in further study that the growth of RA B cells, when cultured both with and without anti-μ, was promoted by IL-1. Thus, it is speculated that B cell-derived IL-1 may be involved in B cell clonal expansion in RA, possibly via an autocrine pathway. As the BCGF activity of IL-1 itself was rather weak in our study, the following possible mechanisms could be considered. First, in RA, the growth of a B cell subset(s) may be promoted directly by IL-1 alone. Recent reports of EBV-transformed B cells exhibiting an autocrine growth through IL-1 (27, 28) lend some support to this. Second, at least as a co-factor, IL-1 can augment B cell response to activation signals mediated by other factors. For instance, IL-1 exerts its effect on B cell growth at the G1/S transition stage subsequent to CD23 expression (25), and in RA, IL-1 enhances the growth and differentiation of anti-CD5 stimulated B cells (29). Third, IL-1, in cooperation with B cell derived-BCGF, may potentiate B cell growth only in areas of high B cell density (30,31). In this regard, B cells frequently form germinal centers within the synovial tissue of RA (1). However, it remains unclear at present whether these BSF activities of IL-1 actually operate in RA.

Our findings on the increased IL-1 production by RA B-cells are not pathognomonic, since Tanaka *et al.* (32) have reported that B-cells from patients with systemic lupus erythematosus produced a large amount of IL-1, IL-4 and IL-6 in the B-cell culture SN. Further study about the ability of IL-1 production by B-cells from other autoimmune disorders would clarify the autocrine mechanism involved in the proliferation of autoantibody producing B-cells.

Acknowledgements. We thank Dr. Yoshikatsu Hirai for providing us with human rIL-1α and β, and monoclonal and polyclonal anti-human IL-1α or β.

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Received July 28, 1990; accepted September 21, 1990.