upon their time, making it more difficult for them to visit a major hospital.

A decline in the severity of RA has been pointed out in Europe and Australia.[1 5 7-9 13] This decline, indicated by seropositivity, radiographic erosions, and rheumatoid nodule positivity has been described by Silman *et al.*[5 7-9] A decline in radiographic severity has also been noted by Heikkilä *et al.*[1] Because, in our study, the patients in group II have a longer disease duration, it could be reasonably assumed that more severe and more patients with longstanding disease were recruited for group II. However, a comparison of data, based on patients with similar disease duration, did not show any significant change over a 30 year period in seropositivity, rheumatoid nodule positivity, and rate of hip involvement, although a decreased rate of hip involvement was observed in group II patients with a disease duration of less than five years.

With regard to the severity of RA, a decrease during the last 30 years was not evident in this limited study. To answer definitely the question of whether the severity of RA has been declining in the Japanese population, it will be necessary to gather and compare additional data in a more extensive, systematic, epidemiological study.

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Enhanced Expression of CD14 Antigen on Myeloid Lineage Cells Derived from the Bone Marrow of Patients with Severe Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** We previously reported the accumulation of abnormal myeloid cell populations reacting with CD14 (MY4) monoclonal antibody in the iliac and epiphyseal bone marrow of patients with severe rheumatoid arthritis (RA). Therefore, we investigated *in vitro* production and modulation of CD14+ myeloid cells from iliac bone marrow cells.

Methods. Mononuclear cells were prepared from iliac bone marrow aspirates from patients with RA. The presence of unusual myeloid cells was assessed by 2 color flow cytometry of cells cultured under various conditions.

Results. Cultured iliac bone marrow cells of patients with severe RA produced 14.7% of CD14+CD15+ cells on average. Cultures derived from healthy donors and from patients with a milder form of RA produced fewer CD14+CD15+ cells (< 10%). The production of CD14+CD15+ cells was enhanced by granulocyte macrophage colony stimulating factor and interleukin 1ß, but inhibited by T lymphocytes.

Conclusion. Production and modulation of CD14+ myeloid cells were observed in iliac bone marrow of patients with severe RA. (*J Rheumatol 1997;***24:465–9**)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
MYELOID LINEAGE CELL
BONE MARROW
CD14 ANTIGEN

Abnormalities in bone marrow of patients with rheumatoid arthritis (RA) suggest that bone marrow might play a role in the pathogenesis of RA[1-5]. We reported the presence of an abnormal myeloid cell population in epiphyseal bone marrow adjacent to joints affected with severe RA[6]. Those abnormal myeloid cells were found to be supported by some soluble factor having myeloid growth activities[7]. These abnormal cells could be distinguished from normal myeloid cells by expression of the difucosyl type 2 chain structure (dimetric Le^x, a specific marker of human undifferentiated cells[8]) or CD14 antigen (MY4 antigen, expressed in mainly monocyte macrophages)[9].

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We investigated cellular changes in iliac bone marrow (one of the major sites of hematopoiesis in the adult) and tibial bone marrow[10]. In the iliac bone marrow of patients with severe RA, the absolute numbers of myeloid lineage cells were increased about 3 times relative to non-RA controls. Moreover, in this increased population of pan-myelocytes, a subset of abnormal myeloid cells (CD14+ myeloid cells) was discovered at higher incidence in iliac bone marrow than in tibial bone marrow in patients with severe RA. This abnormal myeloid cell subset could not be found in iliac and tibial bone marrow in non-RA controls and patients with mild RA. Based upon these findings *in vivo*, we report *in vitro* production and modulation of abnormal (CD14+) myeloid cells from iliac bone marrow of patients with RA.

MATERIALS AND METHODS

Patients. Bone marrow blood samples were obtained from 35 patients with RA (32 women, 3 men) who met the American College of Rheumatology criteria[11] and 8 healthy volunteers (6 women, 2 men), with informed consent. All patients had undergone reconstructive operations at Osaka University Hospital or the related facilities from 1990 to 1993. The average age of patients with RA and controls was 46.9 and 38.5 years, respectively (range 24–59 and 20–55, respectively). Disease activity of all patients was classified as the least erosive subset, the more erosive subset, or mutilating disease, as reported[12]. In this paper, we present "least erosive" as mild disease, and "more erosive" and "mutilating disease" as more severe manifestations of RA.

Cytokines. Recombinant human (rh) interleukin (IL)-1ß was a kind gift from Otuka Pharmaceutical Co. Ltd., Tokushima, Japan. RhIL-3, rhIL-6,

rhIL-8 (monocyte derived), rh monocyte colony stimulating factor (MCSF) and rh granulocyte macrophage colony stimulating factor (GMCSF) were purchased from Genzyme Corporation (Cambridge, MA, USA) and rh granulocyte colony stimulating factor (GCSF) was purchased from Amersham (Amersham, UK).

Monoclonal antibodies. The monoclonal antibodies (Mab) used were fluorescein isothiocyanate (FITC) conjugated CD15 (MX-GA, HL-5) (Kyowa Medix, Tokyo, Japan), phycoerythrin (PE) conjugated CD14 (MY4) (Coulter Immunology, Florida, USA). The CD15 (MX-GA) antibody detects a broad range of myeloid lineage cells from myeloblasts to polymorphonuclear cells[15].

Cell preparations. Heparinized bone marrow aspirate was obtained from anterior iliac crest by needle puncture at the time of operation. Mononuclear cells (MNC) from bone marrow aspirate were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient (1.077 g/ml) centrifugation (30 min, 400 × g). In some experiments, to deplete phagocytic cells, bone marrow aspirates were incubated at 37°C for 1 h with the addition of 1/10 volume of silica suspension (JIMRO, Takasaki, Japan) before Ficoll-Hypaque density gradient centrifugation. T lymphocytes were depleted by incubation with sheep red blood cells (SRBC) (JIMRO, Takasaki, Japan). The cells were then washed 3 times by phosphate buffered saline (PBS).

Cell cultures. Culture medium HL-1 (Ventrex, Portland, ME, USA) was used. Components of HL-1 include ultrapure, pyrogen-free water, a specialized, modified DME: F12 base, HEPES buffer, known amounts of insulin, transferrin, testosterone, sodium selenite, ethanolamine, and stabilizing proteins. Cells were resuspended in HL-1 supplemented with 5% heat inactivated fetal calf serum (Flow Laboratories, North Ryde, Australia) and cultured 5 days at 37°C in a humidified atmosphere of 5% CO_2 at cell densities 0.5–1.0 × 10^6/ml in the presence of iliac bone marrow serum from patients with RA or cytokines, as described.

2-color FACS analysis and cell sorting. The cultured cells were collected at Day 5 and washed 3 times in PBS. The cells were incubated with Mab of FITC conjugated CD15 (MX-GA) and PE conjugated CD14 (MY4) on ice for 30 min, then washed 3 times by PBS and fixed in 1% paraformaldehyde. 2-color flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA, USA) equipped with an argon laser at 488 nm. All specimens were analyzed on the day of collection. To omit debris, analysis was performed on cells selected (gated) by their forward and right angle scatter measurements. The data from 10,000 cells per test were collected and stored in list mode (Consort 30 Hewlett-Packard computer). The percentages of cells positive for either Mab staining were determined by markers formed around isotype and fluorescent matched control antibodies. The number of positive cells was expressed as a percentage of the total cell count. For cell sorting, cells were stained in the same way as for FACS analysis, then sorted on the FACStar Plus (Becton Dickinson). Reanalysis of the sorted cell population was performed and the efficiency of the sorting calculated. Sorting efficiencies exceeded 95% purity.

Statistical analysis. Results were expressed as the mean ± standard deviation. Data were analyzed by nonparametric Mann-Whitney U test or analysis of variance (ANOVA) with post hoc, where appropriate, p values < 0.05 were considered significant.

RESULTS

Production of CD14+ myeloid cells in the iliac bone marrow of patients with RA. To assess *in vitro* production of CD14+ myeloid cells, bone marrow MNC from various donors were incubated 5 days with 10% final concentration of autologous iliac bone marrow serum. In patients with more erosive and mutilating disease, the average ratio of CD14+CD15+ cells among whole MNC was 4.5% (3.3–5.9%) before incubation, but it increased significantly to 14.7% (11.9–17.1%) after a 5 day incubation ($p < 0.0001$). The values assessed from patients with mutilating disease were within the range of those assessed from patients with more erosive disease, and we assessed the values from patients with more erosive and mutilating disease together as more severe RA. In patients with the least erosive disease, the average ratio before incubation was 3.2% (2.8–4.3%), and it increased significantly to 8.1% (6.6–9.8%) after a 5 day incubation ($p < 0.05$). In healthy donors, the average ratio before incubation was 2.4% (1.4–2.8%) and after incubation 3.5% (2.5–3.9%). There was no statistical significance between the ratios before and after incubation in healthy donors (Figure 1).

Effect of T lymphocytes in producing CD14+ myeloid cells. To study the effect of T lymphocytes in producing CD14+CD15+ cells, we compared the ratios of CD14+CD15+ cells among nonphagocytic bone marrow MNC or T lymphocyte depleted nonphagocytic bone marrow MNC of patients with most erosive disease, after incubation for 5 days, in the presence of rhGMCSF (50 U/ml). The cell fractions were as follows: nonphagocytic bone marrow MNC: CD2 50%, CD14 < 3%, CD15 20%, CD34 5%; T lymphocyte depleted nonphagocytic bone marrow MNC: CD2 < 5%, CD14 < 3%, CD15 40%, CD34 15%. From the FACS analysis, the CD14+CD15+ cell numbers produced from 1.0 × 10^5 CD15+ cell were calculated. As shown in Figure 2, the numbers of CD14+CD15+ cells produced in the presence of T lymphocytes and without T lymphocytes were 17,000 ± 4,415 and 33,113 ± 8,162, respectively. In all 4 cases, CD14+CD15+ cells were more numerous cultured without T lymphocytes than in the presence of T lymphocytes ($p = 0.0133$). This result indicates the inhibitory effects of T lymphocytes on production of CD14+CD15+ cells.

Effect of cytokines on production of CD14+ myeloid cells. To measure the effect of various cytokines on production of

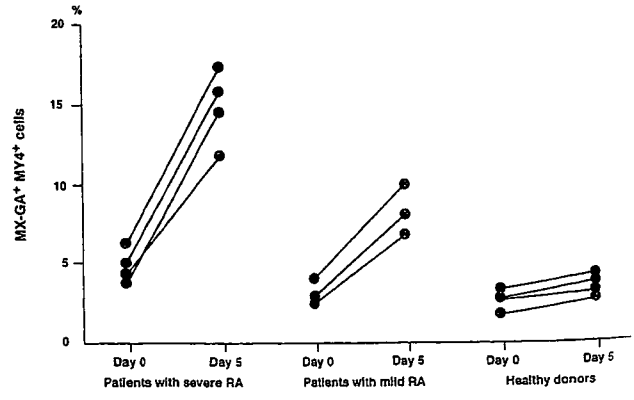

Figure 1. CD14 antigen expression on CD15+ cells. MNC from iliac bone marrow aspirates of patients with severe or mild RA or healthy donors were cultured with auto-iliac serum for 5 days.

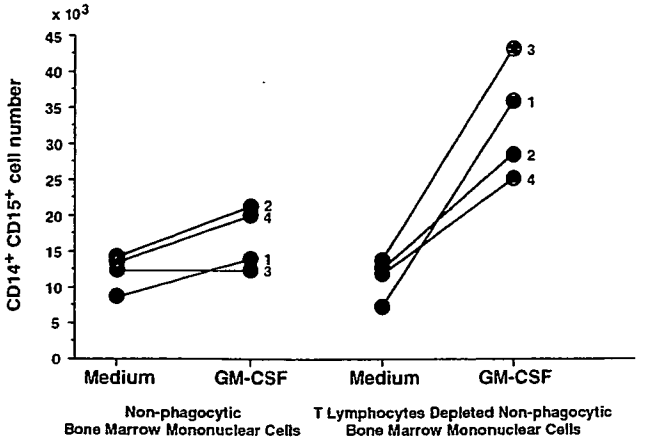

Figure 2. Effect of T lymphocytes on CD14 antigen expression on CD15+ cells from patients with severe RA. Nonphagocytic bone marrow mononuclear cells or T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured in the presence of rhGMCSF (50 U/ml) or without GMCSF for 5 days. Numbers indicate preparations from the same patients. Quantities of CD14+CD15+ cells produced from 1×10^5 CD14–CD15+ cells are shown.

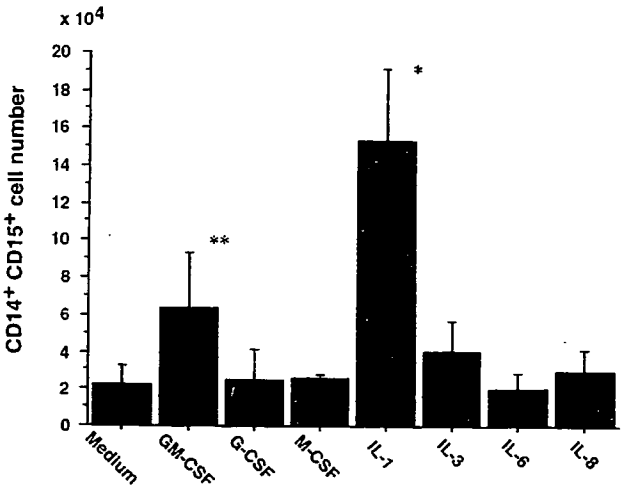

Figure 3. Effect of cytokines on CD14 antigen expression on CD15+ cells from patients with RA. T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured in the presence of cytokines (100 U/ml) for 5 days. *p < 0.0001 and **p = 0.0037 compared with culture with medium only.

CD14+CD15+ cells, T lymphocyte depleted nonphagocytic bone marrow MNC were prepared from a few patients with more erosive disease, and cultured in various concentrations of IL-1ß, IL-3, IL-6, IL-8, GCSF, MCSF, and GMCSF (final concentration of 1, 10, 50, 100, 200, 500 U/ml). With GMCSF and IL-1ß, CD14+CD15+ cells were produced in a dose dependent fashion. The optimal production was achieved with 50 U/ml of GMCSF and 100 U/ml of IL-1ß. So in the following experiments, all cytokines were used at a final concentration of 100 U/ml. T lymphocyte depleted nonphagocytic bone marrow MNC were prepared at cell density 0.5×10^6/ml from 5 patients with more erosive disease and cultured in IL-1ß, IL-3, IL-6, IL-8, GCSF, MCSF, and GMCSF (final concentration 100 U/ml). CD14+CD15+ cells were produced at significantly higher ratios by incubation with IL-1ß and GMCSF than without addition of cytokines ($p = 0.0037$ and $p = < 0.0001$, respectively). No significant enhancement was achieved by adding any other cytokines studied (Figure 3).

Reactivity of CD14– myeloid cells in the iliac bone marrow of patients with RA in the presence of IL-1ß. To compare the reactivity of CD14– myeloid cells in each disease subset, CD14–CD15+ cells obtained by sorting were incubated for 5 days in the presence of IL-1ß (100 U/ml). The average ratio of CD14+CD15+ cells was 8.0 ± 4.2% for healthy donors, 14.1 ± 3.2% for least erosive disease, and 35.6 ± 14.0% for more erosive and mutilating disease, respectively (Figure 4). Thus, the capacity of CD14–CD15+ cells to develop into CD14+CD15+ cells was significantly different between cells derived from more erosive and mutilating disease and from least erosive disease ($p < 0.01$).

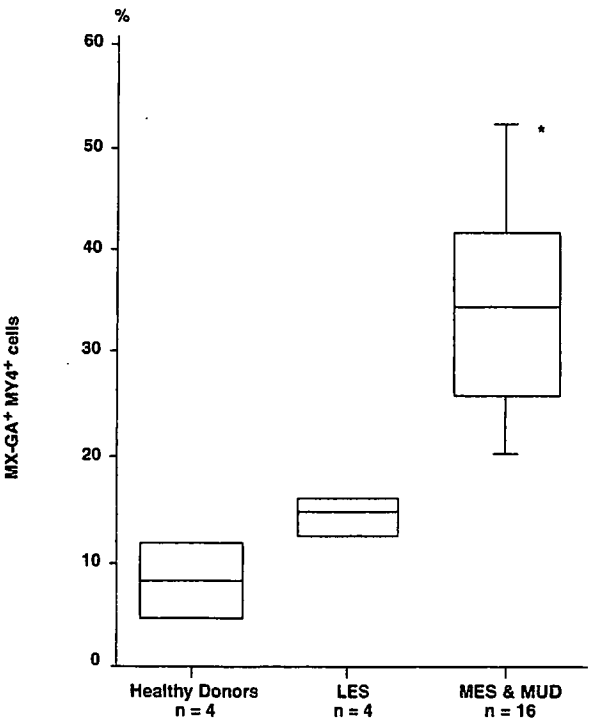

Figure 4. Effect of IL-1ß on CD14 antigen expression on CD15+ cells from patients with RA and healthy donors. T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured 5 days in the presence of IL-1ß (100 U/ml). The line in middle of the box represents the median. The top of the box represents the 75th percentile and the bottom the 25th percentile. *p < 0.01 compared with LES.

DISCUSSION

We previously reported the accumulation of unusual myeloid cell populations bearing oncofetal mono- or difucosylated type 2 chain in epiphyseal bone marrow adjacent to the affected joints in patients with severe RA[6]. These unusual myeloid cells were confirmed to react with CD14 and CD15 Mab[9]. In the present study, these Mab were used to detect unusual myeloid cells. Our aim was to determine the characteristics of these cells. CD14 surface antigen was once thought to be specific to monocyte macrophages and not be expressed on myeloid colony forming cells[14], but a subpopulation expressing low levels of CD14 together with CD16 (FcRIII) was recently identified. CD14 expression can also be observed on primary B cells harvested from blood[15–17]. By repeated morphological analyses of sorted CD14+CD15+ cells, we confirmed those cells to be myeloid cells[10]. Oncofetal membrane marker on the surface of these CD14+CD15+ cells might suggest hyperactivated metabolism in this lineage of cells, but the lineage of these unusual myeloid cells remains unknown.

What is the characteristic function of CD14+CD15+ cells in patients with severe RA? Precise and direct analyses have not yet been done because we have not succeeded in development of these abnormal myeloid cells into polymorphonuclear (PMN) cells (the developed functional cells of myeloid lineage) *in vitro*. We tried to determine whether PMN in the epiphyseal bone marrow of patients with severe RA had some special characteristics. Our previous studies revealed PMN cells with remarkably high levels of IL-1 in the same epiphyseal bone marrow as accumulations of CD14+CD15+ cells[18,19]. IL-1 is known to be a potent stimulator of the synthesis of matrix metalloproteinase by connective tissue cells of joints, such as the synovium, cartilage[20,21], and possibly bone and ligament. Thus, it is possible that highly activated myeloid lineage cells could induce diffuse and severe connective tissue destruction in severe active RA. Another question was how these CD14+CD15+ cells produced and accumulated in iliac bone marrow were transferred to the epiphyseal bone marrow. The peripheral blood circulation is the probable route for these cells to migrate from iliac to epiphyseal bone marrow, as reported by Santiago[22].

The production of unusual (CD14+) myeloid cells from apparently normal (CD14–) myeloid cells was a novel finding. CD14–CD15+ cells in iliac bone marrow from normal donors showed no development to CD14+CD15+ cells even after incubation *in vitro* with high levels of IL-1ß or GMCSF. CD14–CD15+ cells derived from iliac bone marrow of patients with the least erosive disease showed some propensity to develop into CD14+CD15+ cells under the influence of high levels of IL-1ß, albeit at much lower final levels than those of patients with more erosive or mutilating disease. Reactivity of CD14–CD15+ cells in the hematopoietic iliac bone marrow was thought to be quite different relating to severity of RA. Because of the lower reactivity of CD14–CD15+ cells derived from patients with the least erosive disease, CD14+CD15+ cells could not be found in patients with milder RA *in vivo*.

Recently, tumor necrosis factor alpha, transforming growth factor beta, and interferon gamma have been investigated concerning the pathogenesis of RA[23–25], and may have strong effects on enhancing the development of CD14+CD15+ cells. Those factors were not evaluated in our study because we focused on the fundamental pathologic mechanism of development of CD14+CD15+ cells in iliac bone marrow of patients with RA. Among the effects of activating factors in this study, one interesting result is that IL-6 and IL-8 showed no enhancing effect in producing CD14+ myeloid cells. We previously reported elevations of IL-6 and IL-8 levels in iliac bone marrow serum of patients with RA closely related to the remarkable synovial proliferation[26]. It is difficult to draw conclusions from the estimation of cytokine levels in iliac bone marrow, but the mechanisms involved in production of CD14+ myeloid cells may be different from those promoting the proliferation of synovial tissue. Another indication of these differences is the function of T cells. Activation of T cells has been shown to be a fundamental mechanism in induction of synovitis in patients with RA[27], but in this study, T cells were proved to inhibit production of CD14+ myeloid cells. As T lymphocytes have many subpopulations, further work may elucidate the particular subsets of T cells that modulate growth of CD14+ myeloid cells. Studies of the pathophysiology of bone marrow in patients with RA will investigate the roles of various enhancing or inhibitory factors. However, our results suggest that the pathogenesis of RA may involve systemic hematopoietic organs such as iliac bone marrow.

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Phenotypic Characteristics of Bone Marrow Cells in Patients with Rheumatoid Arthritis

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ABSTRACT. Objective. Our previous study showed the presence of abnormal myeloid lineage cells in the epiphyseal bone marrow adjacent to joints affected with severe rheumatoid arthritis (RA). Now, we investigated whether there were any changes of other marrow cell populations related to RA, and whether there were any pathologically characteristic changes in the iliac bone marrow, which is one of the major systemic hematopoietic organs.

Methods. 2-Color flow cytometry was carried out to analyze the phenotypes of mononuclear cells (MNC) fractions in bone marrow aspirates and venous blood from 56 patients with RA and 7 non-RA controls.

Results. The absolute number of MNC in the iliac bone marrow was increased by 3-fold in the RA patients compared with the non-RA controls. In contrast, no significant increase of MNC was observed in the tibial epiphyseal bone marrow or peripheral blood. The ratio of each MNC fraction in the iliac bone marrow did not differ significantly between the RA patients and the non-RA controls. In lymphocyte subsets, the percentage of HLA-DR+CD8+ cells to all CD8 cells in the iliac bone marrow increased significantly in the RA patients compared with the non-RA controls. Abnormal myeloid cells (MX-GA+MY4+ cells), specific to severe RA, were found to be more concentrated in the iliac bone marrow than in the tibial epiphyseal bone marrow.

Conclusion. Characteristic pathologic changes of the iliac bone marrow suggest an important role of systemic bone marrow in the progression of RA. (*J Rheumatol 1994;***21:1608–14**)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
MYELOID LINEAGE CELLS
BONE MARROW

Preceding the induction of polyarthritis, maturation and proliferation of bone marrow cells were observed, and interleukin 1 (IL-1) and IL-6 levels in bone marrow serum were elevated in collagen induced arthritis and adjuvant arthritis in rats[1-3]. These pathological changes were maintained while arthritis continued. The results suggested that bone marrow plays an important role in inducing polyarthritis.

Our studies have demonstrated the existence of abnormal myeloid cells, which strongly express the difucosyl or trifucosyl type 2 chain (dimetric or trimetric Le^x, a specific marker of human undifferentiated cells[4,5]), in the epiphyseal bone marrow adjacent to joints affected with active severe subset of RA[6]. These myeloid lineage cells, which accumulate in the epiphyseal bone marrow in various stages of maturity, were found to exist in severe and active subset of RA. However, these cells were not found in the epiphyseal bone marrow of normal controls, nor in patients with mild RA, osteoarthritis or infectious arthritis. Thus, these myeloid lineage cells appear to be specific for severe RA. Maintaining these cells *in vitro* could not be achieved using commercially available culture medium, but was possible when epiphyseal bone marrow serum from patients with severe RA was added to the medium[7]. These results show the characteristic changes in epiphyseal bone marrow in RA. Recently other investigators have also reported abnormalities in bone marrow in patients with RA[8-10].

These results left us with 2 major questions to be answered: (1) are there any changes in other bone marrow cell populations related to RA and (2) are there any characteristic pathological changes of the iliac bone marrow, which is one of the major sites of systemic hematopoietic organs.

To answer these questions, we studied bone marrow blood cells from a large number of patients with RA. Our test subjects were obtained from the iliac crest and the tibial proxymal epiphysis at the time of surgical procedures.

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MATERIALS AND METHODS

Subjects. Bone marrow blood samples and peripheral blood samples were obtained from 56 patients with RA (51 women, 5 men) who met American College of Rheumatology criteria[11], and 7 healthy volunteers (non-RA controls) (4 women, 3 men) after informed consent was obtained. All the patients were operated on at Osaka University Hospital or the related facilities from 1990 to 1993. The average age of the RA patients and the non-RA controls was 55 and 40 years, respectively (range: 30–75, and 26–55, respectively). The average duration of the disease was 9.3 years (range: 3–30). According to our reported criteria[12,13], we classified patients with RA into 3 disease subsets; the subset with least erosive disease (LES), the subset with more erosive disease (MES) and the subset with mutilating disease (MUD). In the LES, erosive articular changes were primarily limited to the smaller peripheral joints. In the MES, the larger axial joints were also involved. In the most severely affected subset (MUD), which involves mutilating disease, almost all joints were extensively damaged. There were 17 patients in LES, 26 in MES, and 13 in MUD, respectively.

Cell preparations. At the studies of bone marrow cells, peripheral blood contamination of aspirates is thought to be inevitable[14,15], so a preliminary experiment was carried out. We collected iliac bone marrow aspirates in 5 ml fractions up to 30 ml and measured the number of myeloid cells to determine the level of contamination. When the volume aspirated from the iliac bone marrow exceeded 20 ml, the number of myeloid cells began to decrease and a significant difference was recognized due to contamination by peripheral blood (data not shown). Thus, the first 5 ml of bone marrow aspirate could be considered to most closely reflect the number of cells in the iliac bone marrow. So in our study, the first 5 ml of bone marrow aspirate was used for analysis.

Five ml of heparinized bone marrow aspirate was obtained from the iliac crest and the tibial proximal epiphysis by needle puncture at the time of operation. A 5 ml sample of heparinized venous blood was obtained simultaneously. MNC fraction, from bone marrow aspirate and venous blood, were separated by Ficoll-Hypaque density gradient (1.077 g/ml) centrifugation. In MNC fraction of the bone marrow aspirates, a lot of premature cells were contained depending on their density.

Cell staining. For FACS analysis, the cells were washed twice with phosphate buffered saline (PBS) and adjusted to 10^6/ml in RPMI 1640. Then 100 μl of this cell suspension was exposed to 5 μl of antibody (T4, T8, MY4, Leu11c, B1-RD1), or 10 μl of antibody (MX-GA, anti-HLA-DR) for 30 min at 4°C. The cells were then washed 3 times in PBS and fixed with 1% formaldehyde in PBS.

Monoclonal antibodies. The monoclonal antibodies (Mab) used were fluorescein-conjugated T4 (CD4), T8 (CD8), B1-RD1 (CD20) (all from Coulter Immunology Hialeah, FL) and MX-GA (CD15, clone HL5) (from Kyowa Medix, Japan), and phycoerythrin conjugated Leu11c (CD16), anti-HLA-DR (both from Becton Dickinson Mountain View, CA) and MY4 (CD14) (from Coulter Immunology Hialeah, FL).

2-Color flow cytometry. 2-Color flow cytometric analysis was performed using a FACScan (Becton Dickinson Mountain View, CA) equipped with an argon laser at 488 nm. All specimens were analyzed on the day of collection. To exclude debris or dead cells, the cells were gated on the basis of forward and right angle scatter. Each test employed 20,000 MNC and the number of positive cells was expressed as a percentage of the total cell count.

Morphologic studies. For light microscopy, sorted cells suspensions at 10^6/ml in RPMI 1640 were cytospun in 50 μl aliquots onto glass slides for 5 min at 6000 rpm (Shandon Cheshire, England) and stained by the May-Giemza, peroxidase and specific esterase method.

Statistical analysis. Results are expressed as the mean ± standard error of the mean. Data were analyzed by the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

Number of mononuclear cells. The numbers of MNC fraction were measured and the data are shown in Table 1. There was a marked increase in the absolute number of MNC in the iliac bone marrow from patients with RA compared with the non-RA controls. The means (± SEM) of the number of MNC were 3122 ± 225 for patients with RA and 1245 ± 311 for the non-RA controls ($p < 0.01$). The number of MNC was increased according to the severity of RA[12] ($p < 0.05$ for LES and $p < 0.01$ for MES and MUD vs non-RA controls) (Figure 1). In contrast, the absolute number of MNC in the tibial bone marrow and peripheral blood did not differ significantly between the patients with RA and the non-RA controls.

Lymphocyte subsets. We analyzed the ratio of the lymphocyte population by FACScan using CD4 and CD8 Mab as

Table 1. *Cell marker studies on bone marrow aspirates and peripheral blood from 56 patients with RA and 7 non-RA controls*

	Peripheral Blood		Iliac Bone Marrow		Tibial Bone Marrow	
	Controls	RA	Controls	RA	Controls	RA
No. of MNC/mm³	974±167	1401±162	1245±311	3122±225*	952±138	1625±212
Myeloid cells						
% CD15+CD16−	2.9±0.5	6.5±1.5	19.0±3.5	24.4±1.7	6.8±2.0	5.8±0.9
T cells						
% CD4	43.2±3.3	29.5±1.8**	17.4±3.3	17.6±1.1	15.7±6.9	28.0±1.7
% CD8	25.2±2.4	21.2±1.6	20.9±4.4	15.1±0.9	15.1±3.6	23.3±1.5
% DR+CD4+/CD4	10.9±1.8	14.1±1.5	12.7±3.4	18.7±1.6	15.4±2.1	15.3±1.4
% DR+CD8+/CD8	13.7±2.8	27.7±2.1**	14.1±4.1	33.2±2.3**	13.1±2.4	28.8±2.7**
CD4/CD8 ratio	1.84±0.34	1.71±0.17	0.91±0.1	1.2±0.1	0.95±0.20	1.30±0.08
B cells						
% CD20	10.4±2.7	10.3±1.0	10.8±2.4	9.8±0.8	9.3±3.2	13.4±1.8
NK cells						
%CD16	17.3±2.2	15.7±1.6	10.8±2.3	8.2±0.7	8.9±2.0	16.3±1.5

Results are expressed as mean ± SEM.

* $p < 0.01$ as compared with the non-RA controls.

** $p < 0.05$ as compared with the non-RA controls.

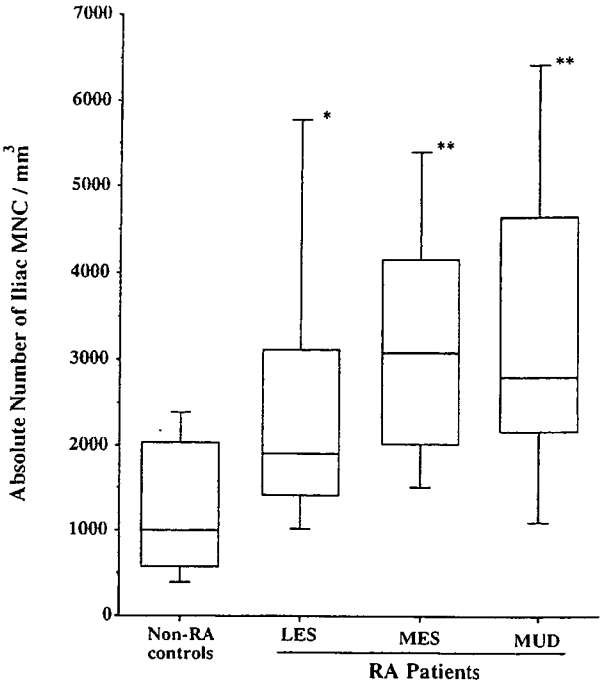

Fig. 1. The absolute number of iliac bone marrow mononuclear cells in non-RA controls and patients with RA. Patients with RA are classified into 3 disease subsets: the subset with least erosive disease (LES), the subset with more erosive disease (MES), and the subset with mutilating disease (MUD). The line in the middle of the box represents the median. The top of the box represents the 75th percentile, and the bottom of the box the 25th percentile. *p <0.05 and **p <0.01 as compared with the non-RA controls.

T cell markers, CD20 as a B cell marker and CD16 as a natural killer cell marker (Table 1). In the iliac bone marrow of patients with RA, the percentages of lymphocytes subsets did not differ significantly from the non-RA controls, but the number of MNC was increased 3-fold compared with the non-RA controls as mentioned above. The epiphyseal bone marrow, on the other hand, showed the similar number and percentage of lymphocytes subsets to those of peripheral blood, and are thought to reflect fundamentally the peripheral circulation. The peripheral blood of patients with RA demonstrated characteristic decrease of CD4 cells compared with the non-RA controls ($p < 0.05$). As to CD4/CD8 ratio there was no significant difference between patients with RA and the non-RA controls in the iliac bone marrow, the tibial bone marrow and the peripheral blood. In general, there were no significant differences in the percentage of lymphocytes in the iliac bone marrow between the patients with RA and the non-RA controls.

HLA-DR positive lymphocytes. We analyzed the percentage of HLA-DR+CD4 and CD8 cells among the total population of CD4 or CD8 cells (Table 1). The percentage of HLA-DR+CD4 cells with respect to all CD4 cells did not differ significantly between patients with RA and the non-RA controls in the iliac bone marrow and peripheral blood. In contrast, the percentage of HLA-DR+CD8 cells to all CD8 cells differed significantly between patients with RA and the non-RA controls in the iliac and tibial bone marrow and peripheral blood ($p < 0.05$). To investigate HLA-DR positive lymphocytes in more detail, we classified the patients with RA into 3 disease subsets[12] and compared each subset with the non-RA controls. The percentage of HLA-DR+CD8 cells to all CD8 cells in the iliac bone marrow was increased significantly in LES and MES when compared with the non-RA controls ($p < 0.05$), whereas the percentage of HLA-DR+CD4 cells to all CD4 cells did not differ significantly between each RA subset and the non-RA controls (Figure 2). Similar differences were observed in the peripheral blood and the tibial bone marrow. In the most severely affected

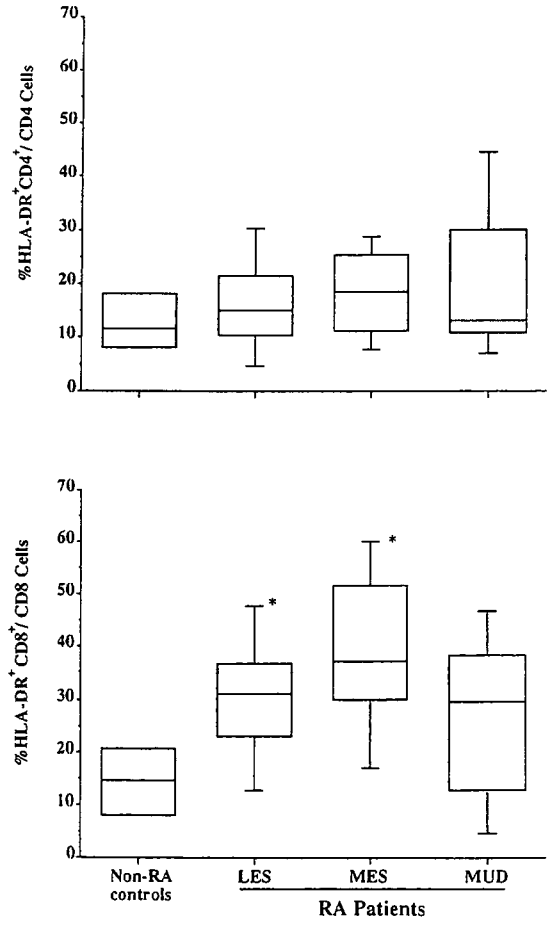

Fig. 2. HLA-DR antigen expression by CD4 and CD8 cells from the iliac bone marrow of the non-RA controls and patients with RA. Patients with RA were as classified in Figure 1. The line in the middle of the box represents the median. The top of the box represents the 75th percentile, and the bottom of the box the 25th percentile. *p <0.05 as compared with the non-RA controls.

patients, MUD, the percentage of HLA-DR+CD8 cells to all CD8 cells varied so greatly that no significant differences could be determined when compared with the non-RA controls.

Myeloid cell population. Myeloid cells were analyzed using the MX-GA (CD15) antibody which detects a broad range of myeloid lineage cells from myeloblasts to polymorphonuclear cells[16,17], and using CD16 which detects mature granulocytes (PMN) and natural killer (NK) cells. In short CD15+CD16− cells were composed of undifferentiated pregranulocyte cells. The CD15+CD16− cells were sorted and cytospun onto glass slides, and stained by the May-Giemza, peroxidase and specific esterase methods. Light microscopy showed that the CD15+CD16− cells were positive for peroxidase and specific esterase staining, and these cells were found to be promyelocytes and metamyelocytes (data not shown). Thus, CD15+CD16− cells were considered to be panmyelocytes.

In the iliac bone marrow, the percentage of myeloid cells among the total MNC fraction showed a clear tendency to increase in patients with RA (Table 1). In addition, the absolute number (/mm^3) of myeloid cells in patients with RA in the 3 disease subsets, differed significantly from the number in the non-RA controls (LES: $p < 0.05$, MES and MUD: $p < 0.01$) (Figure 3).

Abnormal myeloid cells in bone marrow. We tried to analyze the systemic existence of the abnormal myeloid cells found in the involved epiphyseal bone marrow of RA[6]. A preliminary study employing the various monoclonal antibodies showed that abnormal and normal myeloid cells could be well separated using the MY4 (CD14) monoclonal antibody, which reacted with abnormal myeloid cells as well as monocyte-macrophages, but not with normal myeloid cells. In the tibial epiphyseal bone marrow of severe RA (MES and MUD), myeloid cells could be sometimes observed microscopically, and all these cells were recognized as abnormal MY4+ by FACS analysis. On the other hand, no myeloid cells could be found in the tibial epiphyseal bone marrow of the normal controls or LES. It was characteristic that myeloid cells in the iliac bone marrow could be separated into MX-GA+MY4− cells and MX-GA+MY4+ (abnormal) cells in severe RA (MES and MUD). But in the non-RA controls or LES, all myeloid cells in the iliac bone marrow were MX-GA+MY4− (Figure 4). To make clear the population of MX-GA+MY4+ cells in the iliac bone marrow, 2-color flow cytometric analysis was carried out. The purified population of MX-GA+MY4+ cells was selected by sorting, and was examined using cytospin preparations and histochemical stains. As shown in Figure 5, these cells were confirmed to be of myeloid lineage, but not monocyte lineage. In FACS analysis, contamination of counting error must be taken into consideration, and in this study the average background level was 4%. As shown in Table 2, in the tibial epiphyseal bone marrow, the mean percentage (± SEM) of abnormal myeloid cells (MX-GA+MY4+ cells) was almost the same as the background level. There was no significant difference in the patients with severe RA (MES and MUD) when compared with the non-RA controls, although several individual patients in MES and MUD had up to 11.8 and 13.7% of these cells, respectively. So in such cases the abnormal myeloid cell population was recognized. In the iliac bone marrow of patients with more severe RA (MES and MUD), this abnormal myeloid cell population was observed more clearly. In particular, the mean absolute number ± SEM (/mm^3) of these cells in the iliac bone marrow showed a significant difference compared with the non-RA controls ($p < 0.05$). The results are summarized in Table 2.

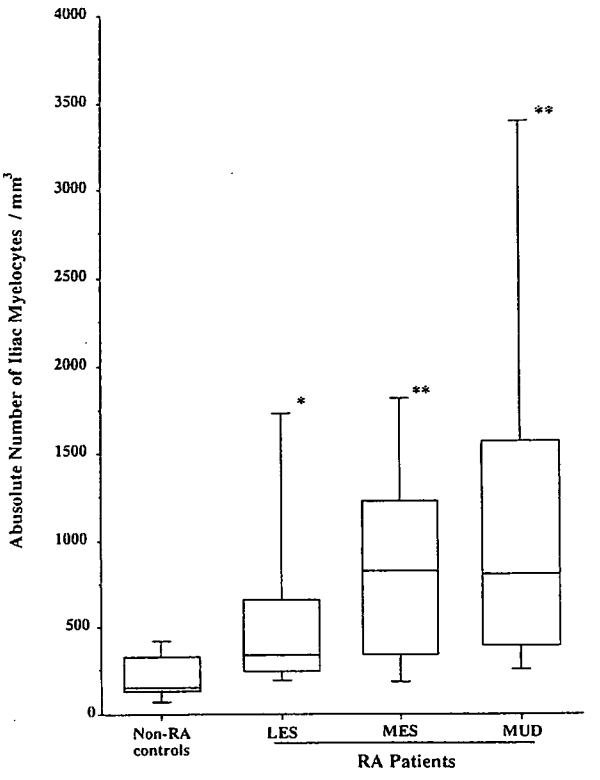

Fig. 3. The absolute number of iliac bone marrow myelocytes (CD15+CD16− cells) in the non-RA controls and patients with RA. Patients with RA were classified as in Figure 1. The line in the middle of the box represents the median. The top of the box represents the 75th percentile, and the bottom of the box the 25th percentile. *p <0.05 and **p <0.01 as compared with the non-RA controls.

DISCUSSION

Our first major question was whether there were any cellular changes in the iliac bone marrow which is one of the major systemic hematopoietic organs in adults. In infants and children, active hematopoiesis takes place in most of the marrow cavities, including the distal long bones. At 5–7 years of age, fat cells begin to replace the hematopoietic marrow in the extremities; in normal adults, hematopoiesis is con-

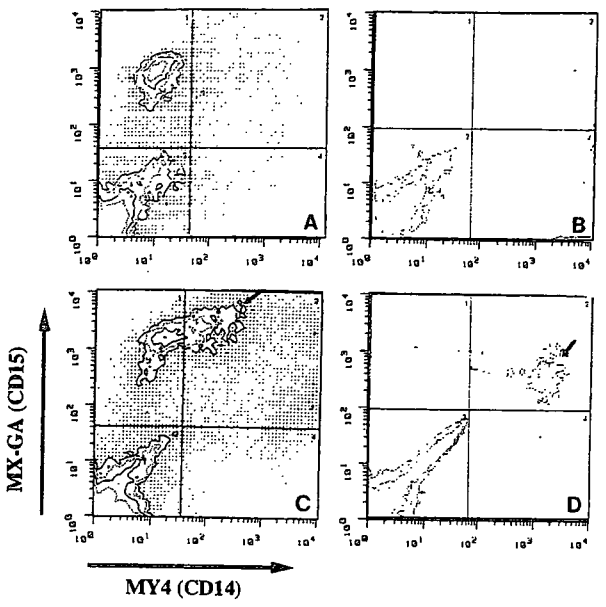

Fig. 4. 2-Color flow cytometric analysis of a normal donor and a patient with severe RA. Panel A: Cells in the iliac bone marrow of a normal donor. Panel B: Cells in the tibial epiphyseal bone marrow of a normal donor. Panel C: Cells in the iliac bone marrow of a patient with severe RA. Panel D: Cells in the tibial epiphyseal bone marrow of a patient with severe RA. In the iliac bone marrow of a normal donor, one myeloid cell population (MX-GA+MY4− cells) was recognized. In the tibial epiphyseal bone marrow, no myeloid cell population was recognized. In contrast, in the iliac bone marrow of a patient with severe RA, two myeloid cell populations (MX-GA+MY4− cells and MX-GA+MY4+ cells) were recognized. And in the tibial epiphyseal bone marrow, an abnormal cell population (MX-GA+MY4+ cells) was recognized. Arrows indicate the abnormal myeloid cell population (MX-GA+MY4+ cells).

fined to the vertebrae, ribs, sternum, pelvis, scapulae, skull, and the extreme proximal portions of the humeri and femora[18,19]. Thus, the tibial epiphyseal bone marrow is not hematopoietic in adults. We chose the iliac bone marrow to investigate hematopoietic marrow in the present study.

The absolute number of MNC (/mm^3) in the iliac bone marrow of patients with RA was increased up to 3-fold compared with the non-RA controls, although the relative proportions of each MNC fraction in the iliac bone marrow was similar between patients with RA and non-RA controls. As there was no significant difference among 3 disease subsets, the increase of MNC was recognized as general to the iliac bone marrow of patients with RA, regardless of the severity of bone and joints destruction. Since the peripheral blood white cell count was not significantly increased in the patients with RA compared with the non-RA controls, the rapid turnover and functional enhancement of white blood cells in peripheral organs of patients with RA was suggested.

One of the characteristic findings in the iliac bone marrow of patients with RA was a significant enhancement of T cell activation. Although the percentage of CD8 cells among MNC was not statistically different between patients with RA and the non-RA controls, the percentage of HLA-DR+CD8 cells to all CD8 cells was significantly higher in both the iliac bone marrow and peripheral blood of patients with LES and MES, but not with MUD. The reason for this is unclear at present, but there could be some fundamental differences of the iliac bone marrow that are unique to MUD. Similar changes were also recognized in peripheral blood but were thought to reflect changes in the iliac bone marrow. The ratio of CD4 cells, on the other hand, was significantly decreased in the peripheral blood as also shown by

Fig. 5. Histochemical staining of purified MX-GA+MY4+ cells. MX-GA+MY4+ cells were stained by the esterase (A) and peroxidase (B) methods (magnification × 800). The morphological findings showed that these MX-GA+MY4+ cells were of the myeloid lineage.

Table 2. *Abnormal myelocytes (MX-GA+MY4+ myelocytes) in iliac and tibial bone marrow*

	n	Percentage in Tibial BM	Percentage in Iliac BM	Absolute Number in Tibial BM (/mm³)	Absolute Number in Iliac BM (/mm³)
Non-RA controls	7	3.7±0.9 (1.5-7.0)	4.4±0.6 (1.8-7.3)	41 ± 13	86 ± 26
LES	17	2.7±0.5 (0.6-7.0)	3.6±0.5 (0.4-8.0)	33 ± 3	96 ± 18
MES	26	4.0±0.6 (0.9-11.8)	6.4±1.3 (1.5-26.0)	102 ± 37	246 ± 79*
MUD	13	4.3±2.0 (1.0-13.7)	7.9±2.3 (1.8-29.5)	94 ± 74	219 ± 65**

Values are the mean ± SEM. Values in parentheses indicate the range. BM: bone marrow.
* $p < 0.05$ vs MES and the non-RA controls. ** $p < 0.05$ vs MUD and the non-RA controls.

prior studies[20,21]. However, the ratio of these cells in the iliac bone marrow was not significantly different to that in the non-RA controls. This could be a result of rapid turnover, and is probably related to the activation of CD4 cells in the peripheral blood. Although the precise pathological mechanisms are not yet known, the activation of T cell subsets could induce various immunologic enhancements such as the elevation of cytokines in the iliac bone marrow. For example, the elevated levels of IL-6 and IL-8 in the iliac bone marrow serum may be related to the remarkable synovial proliferation in multiple joints which is frequently seen in LES and MES[22].

Another characteristic finding in the iliac bone marrow of patients with RA was the induction of abnormal myeloid cells, which were previously reported in epiphyseal bone marrow[6]. In our preliminary study, these cells were clearly distinguished from normal myeloid cells by their antigenic reactivities (MX-GA+MY4+ as well as mono-Lex+di-Lex+ as reported[6]). Because of availability of Mab, in our study these abnormal myeloid cells were defined by staining with MX-GA and MY4. MX-GA+MY4+ cells showed a significant increase in the iliac bone marrow of patients in MES and MUD compared with baseline value found in the non-RA controls. In the tibial epiphyseal bone marrow, there was no significant difference in the percentage or cell number of MX-GA+MY4+ cells between severe RA and the normal controls. This is because the number of these abnormal cells in the epiphyseal bone marrow were reduced during the remission period of RA. In several active cases, however, abnormal myeloid cells were present at up to 11.8% in MES, and 13.7% in MUD, respectively. Thus, abnormal myeloid cells occurred to a greater extent in the iliac bone marrow than in the tibial epiphyseal bone marrow, and was a characteristic feature of MES and MUD. Although the precise physiology and mechanisms involved are still under study, we have reported that polymorphonuclear neutrophils (PMN; the final differentiation stage of myeloids) with a special activity for tissue injury such as PMN factor activity[23,24] and high IL-1 levels[25] were detected in the bone marrow of patients with severe RA (MES and MUD) where those abnormal myeloid cells accumulated.

We have previously analyzed the natural course of RA based on the extent of joint destruction, and reported the existence of 3 disease subsets of RA; LES, MES and MUD[12,13]. We classified these disease subsets according to the number of joints with erosion, serum Clq levels and the annual reduction of the ratio of carpal height[26]. In rough analysis, we found inflammatory variables [C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF)] were persistently remarkably high for at least 5 years after the onset of disease in MES and MUD. Among these inflammatory parameters, CRP levels were persistently above 4 mg/dl within 5 years of onset in MES and MUD, but were usually less than 2 mg/dl in LES. The present study suggested that these clinically defined 3 disease subsets showed significantly different tendencies in the iliac bone marrow cells. That is to say, the increase of HLA-DR+CD8+ T cells in the iliac bone marrow occurs in LES and MES, while the increase of MX-GA+MY4+ cells is characteristic of more severe disease subsets of RA (MES or MUD) (Figure 6). These results suggest that differences at the cellular level affect the natural courses of RA.

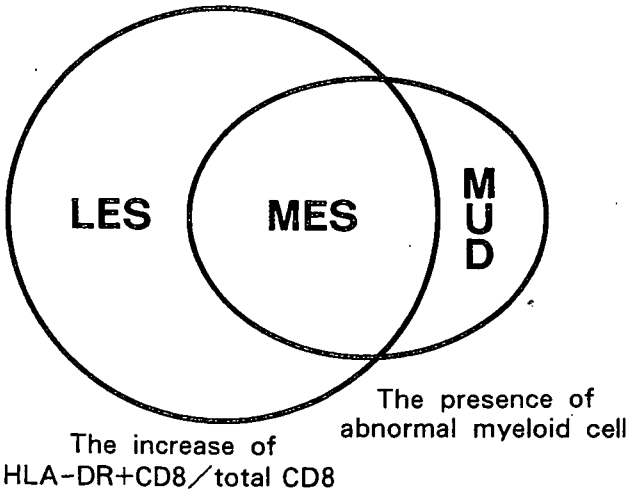

Fig. 6. Characteristic changes of iliac bone marrow cells in each RA disease subset. The increase of the percentage of HLA-DR+CD8 cells to all CD8 cells was recognized in LES and MES, and the presence of abnormal myeloid cells was characteristic to MES and MUD.

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Remarkable Elevation of Interleukin 6 and Interleukin 8 Levels in the Bone Marrow Serum of Patients with Rheumatoid Arthritis

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ABSTRACT. Objective. Characteristic cellular changes have previously been reported in the bone marrow of patients with rheumatoid arthritis (RA). We investigated the levels of various cytokines in RA bone marrow.
Methods. We studied 25 patients with RA (22 women and 3 men) and 10 trauma patients (7 women and 3 men) as non-RA controls. Twelve kinds of cytokines [interleukin (IL)-1α, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, granulocyte colony stimulating factor, granulocyte/macrophage colony stimulating factor, tumor necrosis factor (TNF)-α, and TNF-ß] were assayed by ELISA in iliac bone marrow serum (BMS), tibial BMS, and peripheral blood serum.
Results. Markedly elevated levels of IL-6 and IL-8 were detected in iliac BMS, and much lower levels were found in tibial bone marrow and peripheral blood serum. The levels of IL-6 and IL-8 in iliac BMS showed a close relationship to the extent of synovial proliferation.
Conclusion. Iliac bone marrow may be an important site for the production or accumulation of IL-6 and IL-8 in RA, and these cytokines may influence synovial proliferation in patients with polyarthritis.
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Key Indexing Terms:
BONE MARROW SERUM CYTOKINE RHEUMATOID ARTHRITIS

Earlier we investigated the epiphyseal bone marrow of patients with rheumatoid arthritis (RA) and found abnormal myeloid cells that are possibly related to tissue injury in severe destructive RA[1]. More recently, other characteristic cellular changes of the bone marrow have been reported[2-5]. These findings suggest that the bone marrow cells may play an important role in inducing and/or promoting polyarthritis in patients with RA. In the bone marrow of rats with collagen induced arthritis and adjuvant arthritis, we found that the levels of interleukin (IL)-1α and IL-6 were elevated along with cellular changes[6]. An increase of these cytokines to high levels preceded the induction of arthritis and persisted while the arthritis lasted. Thus, the bone marrow of patients with RA may show changes in soluble factors as well as cellular components.

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Many investigators have described the immunological effects of various soluble factors in patients with RA, including IL-1ß, IL-6, IL-8, and granulocyte/macrophage colony stimulating factor (GM-CSF). These cytokines are believed to be involved in pathogenesis of RA by stimulating synovial proliferation[7], inflammatory cell infiltration[8,9], acute phase protein synthesis[10], and articular cartilage destruction[11-13]. Cellular changes and elevated cytokine levels have been detected in the inflamed synovial tissue and synovial fluid (SF) of RA[14-17]. However, during operations on the joints of patients with severe RA, we sometimes observe that there is little SF accumulation and/or synovial tissue proliferation. Accordingly, we considered the possibility that the bone marrow of patients with RA may also be a site of cytokine production, and thus we investigated the bone marrow levels of various soluble factors.

MATERIALS AND METHODS

Patients. The subjects were 25 patients with RA who satisfied the diagnostic criteria for RA of the American College of Rheumatology[18], and underwent reconstructive surgery at Osaka University Hospital or related hospitals. At the time of surgery, both iliac bone marrow blood and peripheral blood were sampled from all 25 patients and the cytokine levels were measured. Cytokine levels of tibial bone marrow blood could be measured in 15 of these 25 patients. There were 22 women and 3 men with a mean age of 54.7 years (range: 40-70 years) and a mean disease duration of RA of 9.2 years (range: 1-30 years). All the patients had been treated with disease modifying antirheumatic drugs (minimum; for 1 to 9 years), but they still required reconstructive operations for joint destruction. Bone marrow blood was obtained only after the patients gave informed consent. The operated joints were 20 knees, 3 elbows, 4 hands, 1 shoulder, and 1 hip. As

non-RA controls, we also studied 10 trauma patients who had no systemic inflammatory disease or immune abnormalities. They included 7 women and 3 men with a mean age of 56.7 years (range: 42-75 years).

Serum samples. For bone marrow blood samples, we collected 5 ml of nonheparinized blood from the iliac crest and from the proximal tibial metaphysis by needle puncture at the time of operation. At the same time, we collected nonheparinized peripheral venous blood by venipuncture. To obtain the maximum volume of serum, samples were let stand at 37°C for 60 min and then at 4°C for 60 min until the blood was thoroughly clotted. Centrifugation was then done at 1600 × G for 30 min to separate the supernatant, which was stored at −80°C until measurement. A preliminary study confirmed that the levels of cytokines showed no significant difference between blood samples that were processed rapidly or let stand at 37°C for 60 min.

Quantitation of cytokines. The serum levels of 12 different cytokines [IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, granulocyte colony stimulating factor (G-CSF), GM-CSF, tumor necrosis factor (TNF)-α, and TNF-ß] were measured by ELISA (ELISA kit: Quantikine, R&D System), and the concentrations were determined in pg/ml.

Activity of synovitis. At the time of operation, we observed the operated joint and the extent of synovial proliferation was roughly evaluated and classified from grade 0 to 3. Grade 0 indicated no synovial proliferation, while Grade 1 was mild synovitis limited to less than 20% of the joint cavity. Grade 2 indicated moderate synovitis affecting less than half of the joint cavity, and Grade 3 indicated severe synovitis affecting more than half of the joint cavity. There were 6 grade 3 cases, 9 grade 2 cases, 11 grade 1 cases and no grade 0 cases. In addition, 6 patients were unclassified. All of the non-RA controls had grade 0 synovitis.

Laboratory variables. Blood was collected just before the operation and the red blood cell (RBC) count, hemoglobin (Hb) level, white blood cell (WBC) count, % neutrophils (%Neu), and platelet (Plt) count were measured. In addition, the erythrocyte sedimentation rate (ESR), and the levels of rheumatoid factor (RF), immunoglobulin G (IgG), IgA, IgM, and C-reactive protein (CRP) were measured, and the relationships of all these variables with each of the cytokines were studied.

Statistical analysis. Spearman's rank test was used for determining the correlations between the extent of synovitis and the various laboratory variables, while the Wilcoxon signed rank test was used to assess the significance of differences.

RESULTS

Cytokine levels in bone marrow serum. As shown in Figure 1, only the IL-6, IL-8, and G-CSF levels were elevated in bone marrow serum (BMS), except in 3 patients who also had raised IL-1α levels. The other cytokines were close to or below the assay detection limit. Iliac BMS contained IL-6 in 8 of the 25 patients with RA (range: 14–820 pg/ml; median: 46.5 pg/ml), IL-8 in 23 patients (range: 45–7890 pg/ml; median: 200 pg/ml) and G-CSF in 8 patients (range: 17–4200 pg/ml; median: 161 pg/ml) (Figure 1, Table 1). Among the 10 non-RA controls one patient showed a low level of IL-6 (23 pg/ml), and another showed a low level of IL-8 (30 pg/ml). Tibial BMS contained IL-6 in 8 of the 15 patients with RA tested (range: 14–45 pg/ml; median: 18.25 pg/ml). In addition, IL-8 was detected in 9 patients (range 35.5–2300 pg/ml; median: 140.5 pg/ml), and G-CSF

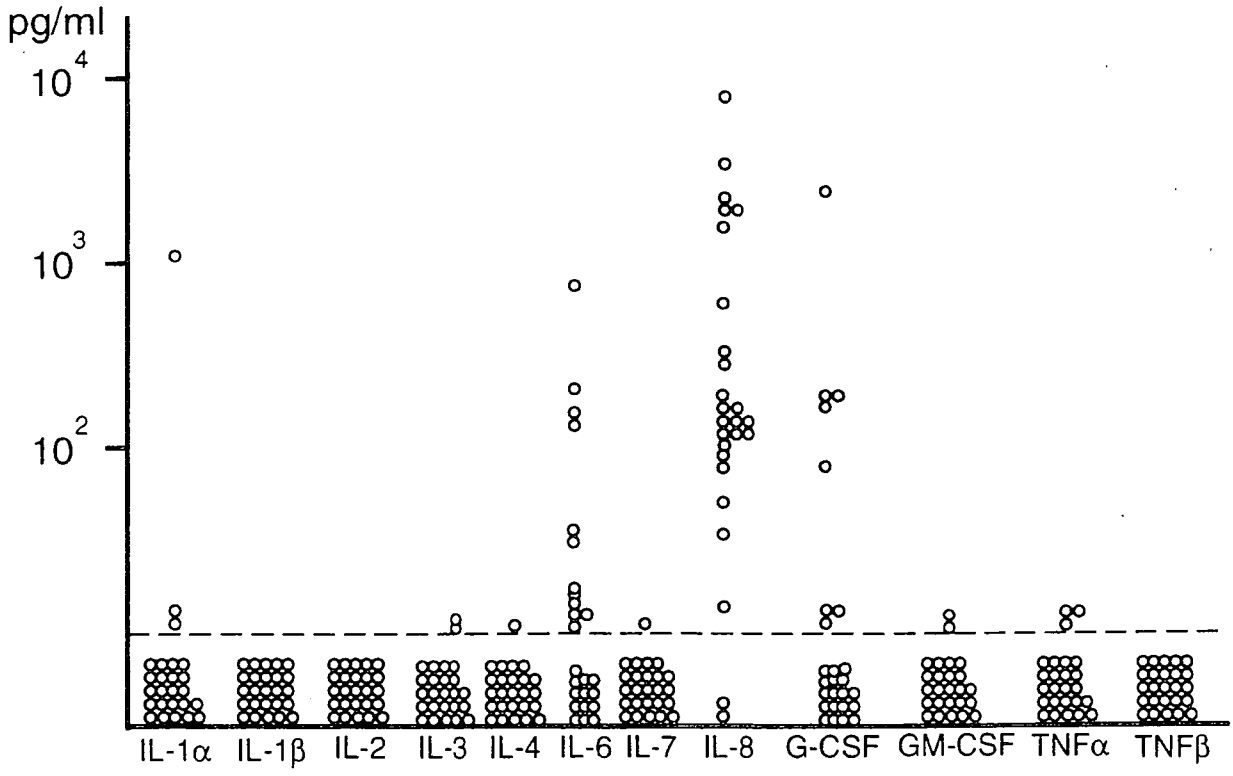

Fig. 1. Levels of IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, G-CSF, GM-CSF, TNF-α, and TNF-ß in bone marrow supernatant from patients with RA. Bone marrow supernatant was obtained as described in Materials and Methods. Cytokine levels were determined with ELISA kits. Values less than the lower limit of detection for each assay are shown beneath the broken line.

Table 1. *Cytokine levels of IL-6, IL-8, and G-CSF in the patients with RA and controls*

	IL-6			IL-8			G-CSF		
	Tibial BMS	Iliac BMS	PBS	Tibial BMS	Iliac BMS	PBS	Tibial BMS	Iliac BMS	PBS
RA									
Number of patients	8/15	8/25	5/25	9/15	23/25	15/25	4/15	8/25	8/25
Range (pg/ml)	14-45	14-820	12.5-75	35.5-2300	45-7890	55-1150	53.5-10,200	17-4200	34-2370
Median (pg/ml)	18.25	46.5	28.5	140.5	200	270	455	161	113.5
Mean (pg/ml)	24.3	158.9	34.6	629.5	1167.6	458.2	2790.9	642.4	654.4
Control									
Number of patients		0/10	1/10		1/10	1/10		0/10	0/10
Range (pg/ml)			23		42	42			
Median (pg/ml)			23		42	42			

*p < 0.05 and ** p < 0.01 (Spearman's rank correlation coefficient).
# p < 0.05 and ## p < 0.01 (Wilcoxon signed rank test).

was found in 4 patients (range: 53.5-10200 pg/ml; median: 455 pg/ml).

Relationship between IL-6, IL-8, and G-CSF in bone marrow serum. We examined whether there were any statistical correlations between the levels of IL-6, IL-8, and G-CSF. The IL-6 level in iliac BMS was closely correlated with the IL-8 level ($p < 0.01$) (Table 1), but the G-CSF was not correlated with either the IL-6 or IL-8 level. In tibial bone marrow, there was no correlation between the levels of IL-6, IL-8, and G-CSF. In addition, there was no correlation between the IL-6, IL-8, and G-CSF levels in iliac bone marrow and those in tibial bone marrow.

Relationships between IL-6, IL-8, and G-CSF in BMS and peripheral blood. The cytokine levels in iliac bone marrow were compared with those in peripheral blood (Figure 2, Table 1). IL-6 was detected in the iliac BMS of 8 patients with RA, the tibial BMS of 8 patients, and the peripheral blood serum (PBS) of 5 patients, with the maximum levels being 820 pg/ml, 45 pg/ml, and 75 pg/ml, respectively. In 6 of 8 patients, the iliac BMS levels were the highest among these 3 samples. The median IL-6 levels were 46.5, 18.25 and 28.5 pg/ml in iliac BMS, tibial BMS, and PBS, respectively, and the iliac BMS level was significantly higher than the PBS level. IL-8 was detected in iliac serum from 23 patients, tibial serum from 9 patients, and peripheral serum from 15 patients, with the highest levels being 7890, 2300, and 1150 pg/ml respectively. In 18 patients the IL-8 levels were highest in iliac BMS. The median IL-8 level was 200 pg/ml in iliac BMS, 140.5 pg/ml in tibial BMS, and 270 pg/ml in PBS, respectively. The median level of iliac BMS included 23 samples which contained 14 cases more than tibial BMS and 8 more than PBS. These additional cases usually showed lower levels and made the median level lower than that of PBS. Although statistical analysis was performed we measured cytokine levels in all 3 lesions, and the levels of iliac BMS were higher than that of PBS.

There were no significant correlations between the IL-6 and IL-8 levels in tibial bone marrow and in peripheral blood. In the case of G-CSF, 2 patients had higher levels and another 2 had lower levels in iliac bone marrow than in peripheral blood (Figure 2).

Relationship of cytokine levels to synovitis. We analyzed the relationship between synovitis and the levels of each cytokine detected in iliac BMS (IL-6, IL-8, and G-CSF). As shown in Figure 3, the levels of both IL-6 and IL-8 increased as synovial proliferation became greater (IL-6: $p < 0.05$; IL-8: $p < 0.005$). In contrast, no correlation was observed between the extent of synovial proliferation and the G-CSF level.

Relationship of cytokines and laboratory variables. To clarify the relationship between bone marrow cytokines and systemic inflammation, we analyzed the correlations with various hematologic variables (CRP, ESR, WBC, %Neu, RBC, Hb, and Plt) and immunologic variables (RF, IgG, IgM, and IgA). No correlations were observed between the levels of IL-6, IL-8, and G-CSF and the laboratory variables. Thus, the elevation of cytokine levels in the bone marrow was not related to systemic inflammation.

DISCUSSION

Our study showed that the IL-6, IL-8, and G-CSF levels were markedly elevated in the iliac bone marrow serum and the peripheral blood of some patients with RA. The levels of IL-6 and IL-8 in many patients were higher in the iliac bone marrow than in the peripheral blood, but G-CSF was more variable and the level was sometimes higher and sometimes lower in the bone marrow than in the peripheral blood. Accordingly we concentrated our investigation on IL-6 and IL-8.

Two possible problems with our experimental procedures need to be considered. One is the dilution of bone marrow blood with peripheral blood at the time of aspiration. In

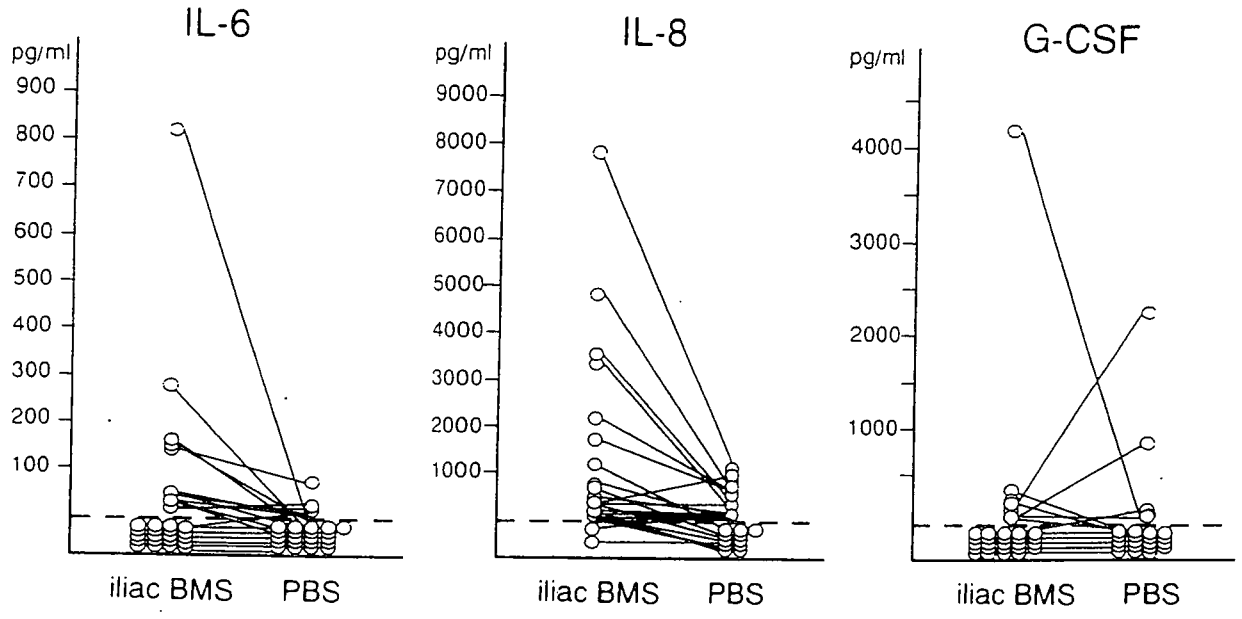

Fig. 2. Comparison of IL-6, IL8, and G-CSF levels between iliac BMS and PBS. Both IL-6 and IL-8 levels in iliac bone marrow were higher than those in peripheral blood (IL-6: $p < 0.05$; IL-8: $p < 0.005$), and the peripheral blood levels increased in proportion with those in iliac bone marrow (IL-6: $p < 0.05$; IL-8: $p < 0.001$). In the case of G-CSF, showed 2 patients had higher levels and another 2 had lower levels in iliac bone marrow than in peripheral blood.

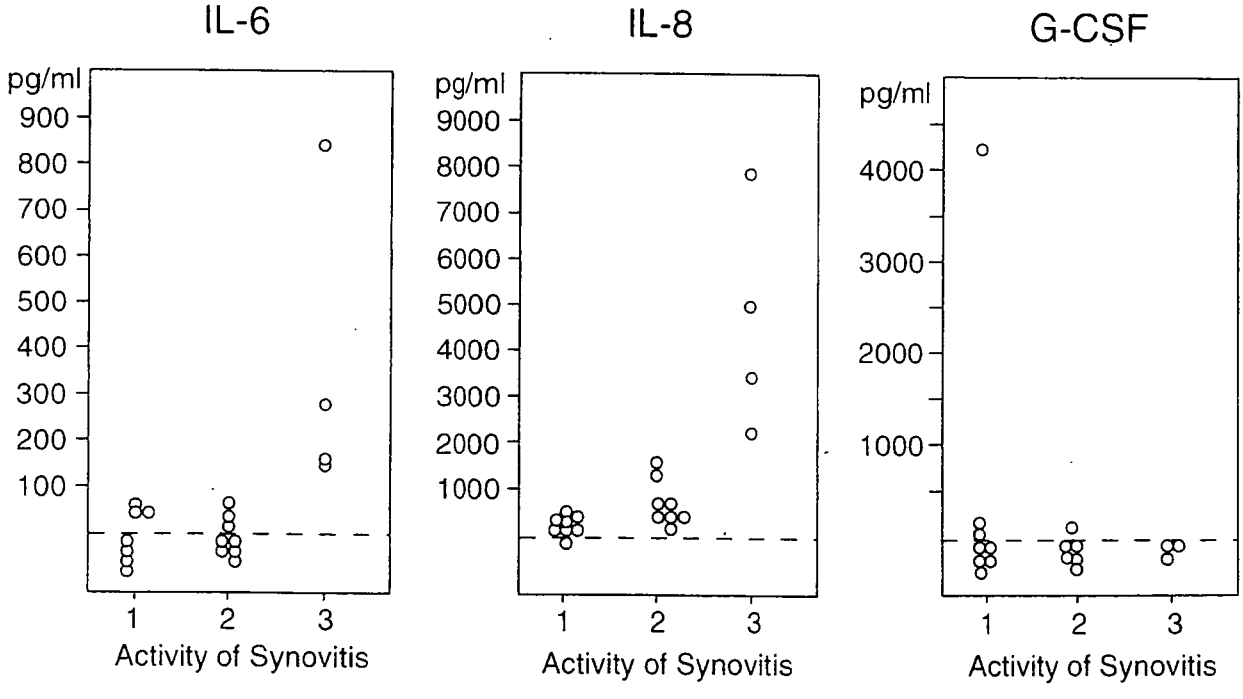

Fig. 3. Relationship between the grade of synovitis and the levels of IL-6, IL-8 and G-CSF. As the extent of synovial proliferation increased, the levels of both IL-6 and IL-8 became higher (IL-6: $p < 0.05$; IL-8: $p < 0.005$). However, no correlation was observed between the extent of synovial proliferation and the G-CSF level.

preliminary experiments, we confirmed that the initial 5 ml of bone marrow blood was minimally diluted with peripheral blood. Thus, the actual IL-6 and IL-8 levels in BMS would be only slightly higher than those reported here. The 2nd potential problem is related to our procedure of leaving the samples at 37°C for 60 min, and at 4°C for 60 min. However, we compared the cytokine levels in serum samples obtained by procedure with those of serum obtained from fresh bone marrow blood, and found no difference.

Elevated levels of IL-6 and IL-8 have been detected in the synovial tissue, SF and peripheral blood of patients with RA in many studies[8,14-17]. In our study, we also measured the levels of IL-6 and IL-8 in the SF of 6 patients. The IL-6 level ranged between 290 pg/ml and 400 pg/ml, while the IL-8 level ranged from 390 pg/ml to 16600 pg/ml. These values were comparable with those reported by other authors[8,16,17]. The cytokine levels in SF were generally higher than those in iliac BMS. However the levels of IL-6 and IL-8 in SF did not show a significant correlation with those in iliac BMS, suggesting that these cytokines were produced independently in the synovial tissue and the iliac bone marrow. Thus, the elevation of these cytokines in synovial tissue reported by other authors could represent a localized mechanism promoting synovial proliferation, while the production of cytokines in iliac bone marrow may represent a systemic mechanism. As the accumulation of SF is not always observed in RA, systemic production of these cytokines could sometimes be important in the exacerbation of synovitis.

The IL-6 and IL-8 levels in PBS of our patients with RA were significantly higher than those in the non-RA controls, but these levels were much lower than those found in iliac BMS or SF, possibly because of dilution or degradation in the peripheral blood.

The epiphyseal bone marrow is a hemopoietic organ in children, but not in adults. However, as myeloid cells including immature cells are found in epiphyseal bone marrow lesions adjacent to the involved joints of adults with severe active RA[2], this bone marrow might regain its infantile hemopoietic function in RA and behave more like iliac bone marrow. In our study, we found that the levels of IL-6 and IL-8 in tibial BMS were not significantly different from those in the peripheral blood. Although higher levels of cytokines occasionally could be found in the tibial BMS, there was no statistical difference in the cytokine levels of epiphyseal bone marrow obtained from bone marrow adjacent to joints with and without synovitis. Our findings suggest that the epiphyseal bone marrow is not a major source of IL-6 and IL-8 like the iliac bone marrow. As there were no significant differences between the cytokine levels in tibial BMS and the peripheral blood, the tibial bone marrow appears to behave differently from the iliac marrow with respect to cytokine production.

The main pathological changes of the synovial villi in RA are known to be synovial cell proliferation, invasion of small blood vessels, and perivascular inflammatory cell infiltration. Elevation of IL-6 and IL-8 levels in the iliac bone marrow and in peripheral lesions could induce the systemic exacerbation of proliferative synovitis in patients with RA, since IL-6 is reported to induce synovial proliferation[7], and IL-8 induces inflammatory cell accumulation[8,9]. IL-6 has also been found to induce osteoporosis by stimulating the proliferation of osteoclasts[19], and IL-8 may play a role in neutrophil mediated cartilage degradation[11]. Although the relationship of these cytokines to bone destruction was not clarified in our study, one of the important mechanisms promoting the exacerbation of synovitis could be elevated levels of IL-6 and IL-8 in iliac bone marrow. Accordingly the disease activity may be controlled systemically by some mechanism in bone marrow.

Despite the correlation between elevated levels of IL-6 or IL-8 and the exacerbation of synovitis in our patients with RA, there was no correlation between these cytokines and laboratory variables of systemic inflammation. This was probably because the levels of acute phase reactants are also influenced by many other factors in RA.

Although IL-1 levels are elevated in the BMS of rats with adjuvant arthritis or with collagen induced arthritis from the early period of the disease[6], the IL-1 levels were not markedly raised in BMS of our patients, except in 3 cases. One patient with an elevated IL-1 level was a 56-year-old man with arthritis of the knee for 6 months, who underwent synovectomy for both diagnostic and therapeutic purposes. After observation for more than 6 months, we diagnosed him as having RA. The other 2 patients were women aged 64 and 63 years with RA for over 5 years. We obtained their iliac BMS at the beginning of an exacerbation of the disease, and found elevated IL-1 levels. At subsequent operations, we were able to obtain their iliac BMS twice more, and found that the IL-1 level was not elevated. Thus, the increase of IL-1 in bone marrow serum could be a phenomenon of short duration occurring with the initiation or exacerbation of RA. In the future, we will continue to investigate how the production and accumulation of cytokines in bone marrow related to intraarticular synovial proliferation and the clinical features of RA.

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教育研修講演

慢性関節リウマチの自然経過と治療計画

(大阪大学医学部環境医学教室)

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Key words : Rheumatoid arthritis, Natural course, A concept for therapy, Disease subsets, Pathology of bone marrow

慢性関節リウマチ(RA)は，何年にもわたり多くの関節の炎症性破壊が進行する慢性進行性疾患である．発症後の経過は軽症に終始する例も，どんどん重症に陥っていく例もあり，さまざまである．このようなRAが発病後10年以上経てどのようになるかの予後はさまざまで，手足の末梢関節のみが侵され日常生活上支障の少ない例から，大関節も侵されて高度の機能障害に陥る例までいろいろある．

しかし，患者さんはRAの診断がつけられると，自分が将来に重症RAに進んで重篤な機能障害に陥ってしまう恐怖感に陥る．またわれわれ臨床家は，目前の患者さんが将来どのようになるかの予測がつかずに困惑するのが実状である．RA患者の長期の経過と予後像を予測することは，患者の長期の治療計画を立てるうえで，また患者に長期の生活設計を立てさせるためにも重要なことであり，われわれはその解析を試みた．その結果，われわれはRAの罹病早期から軽症例，重症例それぞれが別の自然経過をとる，すなわち，病型に分かれていると考えるに至った．そんな疾病であるのに，RAという診断がつくと1つのパターンで金療法を初めとする抗リウマチ剤が考えられるのは不十分ではないか．病態的な差異をも検討し，治療に関しても考察するのが本稿の目的である．

第66回 日本整形外科学会学術集会(神戸)において，教育研修講演として発表した．

I．RAの自然経過

A．早期RA

早期RAの症状は，発熱や全身倦怠感を伴いながら，手のこわばりや，手関節，手指または足趾の関節の明らかな腫れが数週間以上にわたって続く．早期リウマチの診断基準を明確にしようとする活動は，厚生省リウマチ調査研究事業としての研究班[6]や日本リウマチ学会の委員会などで精力的に進められているので，近年中に一定のコンセンサスを得られたものが作られるであろう．

われわれは，手関節や手足指関節などの数関節に慢性に明らかな腫れが続き，赤沈値やCRP値などの炎症反応が明らかな亢進を示し，膠原病や慢性感染症(結核など)などの類似疾患が臨床検査値で否定的なときには，早期RAの可能性が大きいと考えて治療している．

RAが進行してくると，多くは関節にX線的な破壊を認めるようになってくる．やがて誰が診てもRAの状態になり，診断基準[1]を満たして診断が確定されてくる．関節の破壊をX線で認めない程度の例もあり，診断基準でもX線的な関節破壊の画像は必ずしも必要条件ではない．事実，X線的なerosionを認めなくても，手術目的で開けてみると著明な滑膜増殖を認めることはよくある．X線的なerosionもあくまで診断基準の1項目として考えるべきである．当然