Analysis of lymphocyte subsets of bone marrow in patients with rheumatoid arthritis by two colour immunofluorescence and flow cytometry

Minoru Doita, Sakan Maeda, Kazuo Kawai, Kazushi Hirohata, Taketoshi Sugiyama

Abstract

Lymphocyte subsets defined by monoclonal antibodies were investigated in bone marrow and peripheral blood of 17 patients with rheumatoid arthritis (RA); 13 patients with osteoarthritis or aseptic necrosis served as controls. Patients with RA were found to have a raised OKT 4/8 ratio both in bone marrow and peripheral blood in comparison with the controls. Furthermore, bone marrow of RA showed a lower percentage of OKT 8+ T cells than that of controls. The percentage of HLA-DR+ T cells was higher in bone marrow than in peripheral blood of RA, though a slightly lower percentage was detected in bone marrow than in peripheral blood of controls. Thus T cell subsets in bone marrow of RA differ significantly from those of controls. Patients with RA had a higher OKT 4/8 ratio and a higher percentage of HLA-DR+ T cells in bone marrow than controls, suggesting that T cell subsets in bone marrow of RA are in an immunologically activated state and that T cell subsets are affected by rheumatoid inflammation in bone marrow of RA.

Rheumatoid arthritis (RA) is an immunologically mediated disease characterised by a greater amount of immunoglobulin and rheumatoid factors in peripheral blood and synovial fluid,¹⁻³ and by the infiltration predominantly of T cells in synovial fluid⁴⁻⁶ and tissue.⁷⁻¹⁰

The greater number of T cells than B cells in synovial fluid and tissue has emphasised the important role of these cells and the cellular immune response in RA. To understand the chronic inflammatory reactions involved in RA many studies have focused on characterisation of T cell subsets occurring in the tissue and fluids. Reports have indicated variability between patients, and heterogeneity in the distribution of the various subsets. Thus T helper/suppressor ratios have been raised in peripheral blood of RA¹¹⁻¹³ and depressed in synovial fluid.¹⁴⁻¹⁶

Patients with active, persistent RA usually have severe erosion of bone and cartilage also. The synovial pannus burrows into the subchondral bone and greater numbers of lymphoid nodules and plasma cells are also present in bone marrow of RA.¹⁷ An inflammatory reaction commonly occurs in bone marrow of RA, therefore, and interactions among the inflammatory cells may be important in determining the direction of the destructive process. B and T

cells in bone marrow may also be affected through interaction with cells of the synovial pannus and peripheral blood.

To date, the immunohistological distribution of T cell subsets in bone marrow of RA has not been reported. In this paper T cell subsets in bone marrow and peripheral blood of RA were analysed and compared with those in control patients with other forms of joint disease.

Patients and methods

PATIENTS

Seventeen patients (15 female, two male, aged 34 to 72) with classic RA, as defined by the American Rheumatism Association criteria,¹⁸ participated in the study. Twelve patients with osteoarthritis and one with aseptic necrosis (11 female, two male, aged 44 to 82) served as controls. None of the subjects had received steroids or cytotoxic drugs. The bone marrow samples were obtained during the joint replacement.

CELL PREPARATION

At the time of total joint replacement (knee or hip bone marrow cells were aspirated aseptically from the femoral trunk adjacent to the joint. Samples were diluted in RPMI 1640 medium (Nissui Pharmaceutical Co, Tokyo) supplemented with antibiotics and centrifuged at 300 g for 10 minutes. The cell pellets were washed twice in phosphate buffered saline (PBS) and resuspended in 10 ml of PBS.

Heparinised blood (10 ml) obtained by venepuncture at the time of bone marrow aspiration, was diluted with an equal volume of PBS. Each sample was layered on a Ficoll-Hypaque density gradient¹⁹ and centrifuged at 400 g for 30 minutes. Cells at the interface were collected, washed twice with PBS, and adjusted to a concentration of 5×10^6 cells/ml.

REAGENTS

Table 1 shows the monoclonal antibodies used in this study. OKT 3, OKT 4, and OKT 8 were purchased from Ortho Diagnostic Systems (Raritan, New Jersey). Leu 2 and Leu 4 (both fluorescein isothiocyanate (FITC) conjugated) and Leu 15 and HLA-DR (both phycoerythrin conjugated) were purchased from Becton Dickinson (Sunnyvale, California).

INDIRECT IMMUNOFLUORESCENT STAINING

Aliquots (200 μ l) of cell suspension containing

Department of
Orthopaedic Surgery,
Kobe University School
of Medicine, Kobe,
Japan

M Doita
K Kawai
K Hirohata

Department of
Pathology, Kobe
University School of
Medicine, Kobe, Japan
S Maeda
T Sugiyama

Correspondence to:
Dr Minoru Doita,
Department of Orthopaedic
Surgery, Kobe University
School of Medicine, 7-5-1
Kusunoki-cho, Chuo-ku,
Kobe, Japan.

Accepted for publication
2 June 1989

Table 1: Specificity of monoclonal antibodies used in this study

Marker	Specificity
OKT 3	Peripheral T cells
Leu 4	Peripheral T cells
OKT 4	Helper/inducer T cell subsets
OKT 8	Suppressor/cytotoxic T cell subsets
Leu 2a	Suppressor/cytotoxic T cell subsets
Leu 15	Broad cell distribution including granulocytes, monocytes and NK cells: Leu 2+ 15+ subset containing suppressor T cell population
HLA-DR	DR (L243)

1×10^6 cells were incubated at 4°C for 30 minutes with 5 µl of monoclonal antibody, then washed twice with PBS. The cells were then incubated with a 1/80 diluted FITC conjugated goat F(ab)₂ antimouse IgG (Ortho Diagnostic Systems) for an additional 30 minutes. The cells were washed twice and resuspended in 1 ml of RPMI 1640 for flow cytometry analysis.

TWO COLOUR DIRECT IMMUNOFLUORESCENT STAINING

Similar cell suspensions were incubated at 4°C for 30 minutes with a mixture of FITC conjugated Leu 2 and phycoerythrin conjugated Leu 15 to detect suppressor T cell subsets,²⁰ and with a mixture of FITC conjugated Leu 4 and phycoerythrin conjugated HLA-DR to detect activated T cell subsets. After washing twice in PBS they were resuspended in 1 ml of RPMI 1640 for flow cytometry analysis.

FLOW CYTOMETRY ANALYSIS

In single parameter fluorescence experiments the percentage of positively fluorescent lymphocytes was measured on an Ortho Spectrum III laser flow cytometer (Ortho Instruments, Westwood, MA).

In two colour immunofluorescence experiments with FITC and phycoerythrin conjugates 488 nm light (30 mW, argon laser) was used to excite both fluorochromes. The double fluorescence signals from each cell were detected by separate photomultiplier tubes. Data on individual cells were collected, stored, displayed, and analysed by Data Handling System 2140 (Ortho Instruments).

STATISTICAL ANALYSIS

The significance of differences in lymphocyte subset percentages between RA and controls was calculated by Student's *t* test. The Wilcoxon matched pairs signed rank test was used to assess differences between peripheral blood and bone marrow ratios of Leu 4+ HLA-DR+ to total Leu 4+ cells.

Results

EXPRESSION OF OKT AND Leu MARKERS

Table 2 shows that the percentage of cells bearing all markers (OKT 3+, 4+, 8+) was smaller in RA than controls, both in bone marrow and peripheral blood. The difference was significant for OKT 8+ cells in bone marrow ($p < 0.01$). This gave an OKT 4/8 ratio for RA bone marrow and blood significantly greater than for controls ($p < 0.05$). The OKT 4/8 ratio was smaller in bone marrow than in blood for both RA and control groups ($p < 0.001$).

The OKT 8+ T cell subset contains cytotoxic (Leu 2+ 15-) as well as suppressor (Leu 2+ 15+) T cells. Table 2 shows that both subpopulations were smaller in RA than in controls (bone marrow and peripheral blood), though the individual differences were not significant.

Another interesting trend was that regardless of the type of disease Leu 2+ 15+ subsets were more populous in peripheral blood than bone marrow (table 2). The opposite was true for Leu 2+ 15- cells. Cytotoxic (Leu 2+ 15-) predominated over suppressors in all cases ($p < 0.001$). Thus the ratio Leu 2+ 15+/Leu 2+ 15- was smaller for bone marrow than peripheral blood ($p < 0.001$), but differences between the RA and control groups were not significant.

EXPRESSION OF HLA-DR (Ia-LIKE) ANTIGEN

The figure compares ratios of Leu 4+ HLA-DR+ to total Leu 4+ cells. In patients with RA taken as a group, the ratio was significantly greater in bone marrow than in peripheral blood ($p < 0.05$, Wilcoxon matched pairs signed rank test). There was marked heterogeneity, however, as in two of the 11 patients with RA studied the ratios were almost identical in bone marrow and peripheral blood. The control group was also heterogeneous; seven of the nine patients had greater ratios in peripheral blood than in bone marrow, whereas the other two had lower ratios. When the patients were

Table 2: Prevalence of T cell markers in cells from peripheral blood and bone marrow. * Values are given as means (SD)

Marker	Peripheral blood			Bone marrow		
	RA	Controls	All groups	RA	Controls	All groups
OKT 3	62.8 (11.2)	67.3 (11.4)		37.4 (15.3)	42.4 (10.1)	
OKT 4	40.2 (8.2)	40.3 (8.6)		18.5 (7.7)	17.7 (6.2)	
OKT 8	21.9 (8.3)	25.8 (11.2)		19.5 (7.7)†	27.9 (4.8)†	
OKT 4/8 ratio	2.06 (0.82)‡	1.58 (0.54)‡	1.82 (0.73)¶	1.01 (0.45)§	0.63 (0.26)§	0.84 (0.42)¶
Leu 2+ 15+	7.7 (3.9)	8.2 (3.5)		4.0 (2.8)	4.8 (2.4)	
Leu 2+ 15-	15.1 (6.7)	14.6 (7.3)		19.9 (8.5)	22.1 (6.3)	
Leu 2+ 15+/Leu 2+ 15- ratio	0.57 (0.37)	0.63 (0.32)	0.60 (0.34)¶	0.20 (0.10)	0.25 (0.17)	0.22 (0.14)¶

*Cells carrying the individual markers expressed as percentage of all cells.

Pairs of means shown to be significantly different are marked with the same superscript. † $p < 0.01$; ‡ $p < 0.05$; ¶ $p < 0.001$.

Comparison of ratios of Leu 4+HLA-DR+ to total Leu 4+ cells in peripheral blood (PB) and bone marrow (BM).

considered as a group, however, the difference between control blood and bone marrow ratios was not significant. When groups with RA and control groups were compared directly (figure) there was a trend (not statistically verified) for bone marrow HLA-DR+ cells to be increased in RA, when expressed as a percentage of total Leu 4+ cells.

Discussion

Reports of T helper/suppressor ratios in the peripheral blood of patients with RA vary from normal^{21 22} to raised owing to relative reduction of the OKT 8+ suppressor/cytotoxic subset.¹¹⁻¹³ Variability is probably due to the broad clinical spectrum of cases that may be classified as RA. A similar variability is also apparent in RA synovium, depending on the location of the synovial biopsy. It is generally accepted, however, that reduction in the OKT 8+ subset accompanies active RA. This is inversely correlated with an increase of serum C reactive protein and rheumatoid factor.¹⁴ Taken together these may be considered as evidence of immune hyperactivity and progressive joint destruction. In this study a tendency for increased OKT 4/8 ratios in peripheral blood of patients with RA was also found ($p < 0.05$).

T cell subsets in bone marrow, though considered part of the circulating pool,²³ do not necessarily have population ratios similar to those in peripheral blood. In normal marrow OKT 8+ cells predominate over OKT 4+.²³⁻²⁵ Also, within the OKT 8+ subset cytotoxic cells (Leu 2+ 15-) predominate over suppressors (Leu 2+ 15+) to a great degree than in peripheral blood. Our results show that the OKT 4/8 ratio is less in bone marrow than in

peripheral blood both in patients with RA and in controls ($p < 0.001$). The significant disease related finding was the greater increase of the OKT 4/8 ratio in patients with RA than in controls, both in bone marrow and peripheral blood ($p < 0.05$). Furthermore, a suppression of OKT 8+ cells in the marrow of patients with RA compared with controls ($p < 0.01$) was also found. From table 2 it seems that both Leu 2+ 15- and Leu 2+ 15+ subsets of OKT 8+ cells were diminished in RA in comparison with controls.

One may speculate that suppression of OKT 8+ cells in rheumatoid bone marrow is connected with invasion of subchondral bone by hypertrophic synovial pannus. This was seen in all our patients. It is conceivable that lymphocytes and macrophages within the synovial tissue may modulate the bone marrow T cell populations by secretion of biologically active mediators.²⁶⁻³⁰ This may run parallel to the appearance of greater numbers of lymphoid nodules and plasma cells in marrow which have been associated with invasion by pannus.¹⁷

The HLA-DR+ surface antigen is a marker of immune activation in T cells.³ DR+ T cells are seen in rheumatoid synovial membranes³¹ and synovial fluids,^{14 15 21 22 32} and our data also indicated an increase of bone marrow DR+ cells associated with RA. It is not known whether this is a local phenomenon associated with invasion by rheumatoid pannus, or whether there is a widespread increase of bone marrow DR+ T cells owing to systemic influences in RA.

In summary, markers of disease activity in RA, such as increase of the OKT 4/8 ratio, comparative reduction in OKT 8+, and increase of DR+ T cells, were found in bone marrow adjacent to severely affected joints that featured invasion of pannus into subchondral bone. There was considerable variability between individual patients, however, and the significance of the findings in relation to the pathogenesis of RA remains unclear.

The authors wish to thank members of the Kansai Medical Laboratory. Flow cytometry was expertly performed by S Araya and H Arai.

- Koopman W J, Schronhenloher R E. Enhanced in vitro synthesis of IgM rheumatoid factor in rheumatoid arthritis. *Arthritis Rheum* 1980; 23: 985-92.
- Wernick R, LoSpalluto J J, Fink C W, Ziff M. Serum IgG and IgM rheumatoid factors by solid phase radioimmunoassay. *Arthritis Rheum* 1981; 24: 1501-11.
- Winchester R J, Kunkel H G. The human Ia system. *Adv Immunol* 1979; 28: 221-9.
- Froland S S, Natvig J B, Husby G. Immunological characterization of lymphocytes in synovial fluid from patients with rheumatoid arthritis. *Scand J Immunol* 1973; 2: 67-72.
- Sheldon P J, Papamichail M, Holborow E J. Studies on synovial fluid lymphocytes in rheumatoid arthritis. *Ann Rheum Dis* 1974; 33: 509-14.
- Utsinger P D. Synovial fluid lymphocytes in rheumatoid arthritis. *Arthritis Rheum* 1975; 18: 595-602.
- Abrahamsen T G, Froland S S, Natvig J B, Pahl J. Elution and characterisation of lymphocytes from rheumatoid inflammatory tissue. *Scand J Immunol* 1975; 4: 823-9.
- Bankhurst A D, Husby G, Williams R C Jr. Predominance of T cells in the lymphocyte infiltrates of synovial tissues in rheumatoid arthritis. *Arthritis Rheum* 1976; 19: 555-63.
- Loewi G, Lance E M, Reynolds J. Study of lymphoid cells from inflamed synovial membranes. *Ann Rheum Dis* 1975; 34: 524-9.
- Van Bozel J A, Paget S A. Predominantly T-cell infiltrate in synovial membranes. *N Engl J Med* 1975; 293: 517-9.

- 11 Duclos M, Zeidler H, Liman W. Characterization of blood and synovial fluid lymphocytes from patients with rheumatoid arthritis and other joint disease by monoclonal antibodies (OKT series) and acid alpha-naphthylesterase staining. *Rheumatol Int* 1982; 2: 75-82.
- 12 Fox R I, Fong S, Sabharwal N, Carstens S A, Kung P C, Vaughan J H. Synovial fluid lymphocytes differ from peripheral blood lymphocytes in patients with rheumatoid arthritis. *J Immunol* 1982; 128: 351-4.
- 13 Veys E M, Hermanns P, Schindler J. Evaluation of T cell subsets with monoclonal antibodies in patients with rheumatoid arthritis. *J Rheumatol* 1982; 9: 25-9.
- 14 Bertouch J V, Roberts-Thomson P J, Brooks P M, Bradley J. Lymphocyte subsets and inflammatory indices in synovial fluid and blood of patients with rheumatoid arthritis. *J Rheumatol* 1984; 11: 754-9.
- 15 Duke O, Panayi G S, Janossy G, Poulter L W, Tidman A N D N. Analysis of T cell subsets in the peripheral blood and synovial fluid of patients with rheumatoid arthritis by means of monoclonal antibodies. *Ann Rheum Dis* 1983; 42: 357-61.
- 16 Nilsson E, Biberfeld G. T lymphocyte subpopulations defined by monoclonal antibodies in synovial fluid of patients with rheumatoid disease. *J Clin Lab Immunol* 1982; 9: 93-7.
- 17 Sokoloff L, Hough A J Jr. Pathology of rheumatoid arthritis and allied disorders. In: McCarty D J, ed. *Arthritis and allied conditions*, 10th ed. Philadelphia: Lea and Febiger, 1985: 571-92.
- 18 Ropes M W, Bennett G A, Cobb S, Jacox R, Jessar R A. Revision of diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 1958; 9: 175-6.
- 19 Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; 21 (suppl 97): 1-109.
- 20 Clement L T, Dagg M K, Landy A. Characterization of human lymphocyte subpopulations: alloreactive cytotoxic T-lymphocyte precursor and effector cells are phenotypically distinct from Leu 2+ suppressor cells. *J Clin Immunol* 1984; 4: 395-402.
- 21 Burmester G R, Yu D T Y, Irani A M, Kunkel H G, Winchester R T. Ia+ T cells in synovial fluid and tissue of patients with rheumatoid arthritis. *Arthritis Rheum* 1981; 24: 1370-6.
- 22 Forre O, Dobloug J H, Natvig J B. Augmented numbers of HLA-DR-positive T lymphocytes in the synovial fluid and synovial tissue of patients with rheumatoid arthritis and juvenile rheumatoid arthritis: in vivo-activated T lymphocytes are potent stimulators in the mixed lymphocyte reaction. *Scand J Immunol* 1982; 15: 227-31.
- 23 Janossy G, Tidman N, Papageorgiou E S, Kung P C, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. *J Immunol* 1981; 126: 1608-13.
- 24 Abdou N L, Alavi J B, Abdou N I. Human bone marrow lymphocytes: B and T cell precursors and subpopulations. *Blood* 1976; 47: 423-30.
- 25 Janossy G, Tidman N, Selby W S, et al. Subpopulations of human T lymphocyte occupy different microenvironments. *Int J Immunopharmacol* 1981; 3: 209-29.
- 26 Egeland T, Mellbye O J, Pahle J A. Inflammatory synovial tissue mononuclear cells release leucocyte migration factor in antigen- and mitogen-free cultures. *Ann Rheum Dis* 1981; 40: 388-95.
- 27 Klareskog L, Forsum U, Scheynius A, Kabelitz D, Wigzell H. Evidence in support of a self-perpetuating HLA-DR-dependent delayed-type cell reaction in rheumatoid arthritis. *Proc Natl Acad Sci USA* 1982; 79: 3632-6.
- 28 Nathan C F, Murray H W, Cohn Z A. The macrophage as an effector cell. *N Engl J Med* 1980; 303: 622-6.
- 29 Stastny P, Rosenthal M, Andreis M, Ziff M. Lymphokines in the rheumatoid joint. *Arthritis Rheum* 1975; 18: 237-43.
- 30 Wahl S, Gately C L. Modulation of fibroblast growth by a lymphokine of human T cell and continuous T cell line origin. *J Immunol* 1983; 130: 1226-30.
- 31 Kurosaka M, Ziff M. Immunoelectron microscopic study of the distribution of T cell subsets in rheumatoid synovium. *J Exp Med* 1983; 158: 1191-210.
- 32 Kato K, Okada J, Kashiwazaki S. The role of Ia positive T cells in patients with rheumatoid arthritis. *Rheumatol Int* 1986; 6: 1-5.