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, and by the infiltration predominantly of T cells in synovial fluid and tissue.

The greater number of T cells than B cells in synovial fluid and tissue has emphasised the importance 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 RA and depressed in synovial fluid.

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. 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
PATTERNS
Seventeen patients (15 female, two male, aged 34 to 72) with classic RA, as defined by the American Rheumatism Association criteria, 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 gradient 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 ×10⁶ 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 μl) of cell suspension containing
1×10⁶ 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.

**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.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT 3</td>
<td>Peripheral T cells</td>
</tr>
<tr>
<td>Leu 4</td>
<td>Peripheral T cells</td>
</tr>
<tr>
<td>OKT 4</td>
<td>Helper/inducer T cell subsets</td>
</tr>
<tr>
<td>OKT 8</td>
<td>Suppressor/cytotoxic T cell subsets</td>
</tr>
<tr>
<td>Leu 2a</td>
<td>Suppressor/cytotoxic T cell subsets</td>
</tr>
<tr>
<td>Leu 15</td>
<td>Broad cell distribution including granulocytes, monocytes and NK cells: Leu 2+ 15+ subset containing suppressor T cell population</td>
</tr>
<tr>
<td>HLADR</td>
<td>DR (L243)</td>
</tr>
</tbody>
</table>

**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)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Peripheral blood</th>
<th>Bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RA</td>
<td>Controls</td>
</tr>
<tr>
<td>OKT 3</td>
<td>62.8 (11.2)</td>
<td>67.3 (11.4)</td>
</tr>
<tr>
<td>OKT 4</td>
<td>40.2 (8.2)</td>
<td>40.3 (8.6)</td>
</tr>
<tr>
<td>OKT 8</td>
<td>21.9 (8.3)</td>
<td>25.8 (11.2)</td>
</tr>
<tr>
<td>OKT 4/8 ratio</td>
<td>2:06 (0.82)</td>
<td>1:58 (0.54)</td>
</tr>
<tr>
<td>Leu 2+</td>
<td>15+</td>
<td>7.7 (3.9)</td>
</tr>
<tr>
<td>Leu 2+</td>
<td>15+</td>
<td>15.1 (6.7)</td>
</tr>
<tr>
<td>Leu 2+ 15−</td>
<td>0.57 (0.37)</td>
<td>0.63 (0.32)</td>
</tr>
</tbody>
</table>

*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\(^1\) to raised owing to relative reduction of the OKT 8+ suppressor/cytotoxic subset.\(^1\)\(^-\)\(^3\) 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.\(^4\) 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, do not necessarily have population ratios similar to those in peripheral blood. In normal marrow OKT 8+ cells predominate over OKT 4+.\(^2\)\(^-\)\(^5\) 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.\(^2\)\(^-\)\(^6\)\(^-\)\(^3\) This may run parallel to the appearance of greater numbers of lymphoid nodules and plasma cells in marrow which are associated with invasion by pannus.\(^17\)

The HLA-DR+ surface antigen is a marker of immune activation in T cells.\(^5\) DR+ T cells are seen in rheumatoid synovial membranes\(^1\)\(^-\)\(^3\) and synovial fluids,\(^6\)\(^-\)\(^9\)\(^12\)\(^-\)\(^13\) 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.
T cell subsets in bone marrow of RA

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. Rheumatology 1982; 2: 75-82.


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