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Comparative analysis of CD45RA- and CD45RO-positive $CD4^{+}$T cells in peripheral blood, synovial fluid, and synovial tissue in patients with rheumatoid arthritis and osteoarthritis

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Comparative analysis of CD45RA- and CD45RO-positive $CD4^+$T cells in peripheral blood, synovial fluid, and synovial tissue in patients with rheumatoid arthritis and osteoarthritis*

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Abstract

 To determine whether the predominant infiltration with memory $CD4^+$T cells in joints is specific to the local immune and inflammatory response in rheumatoid arthritis (RA), the proportions of $CD45RA^+$ or $CD45RO^+$ cells in the $CD4^+$T cell populations in three different compartments (i.e., peripheral blood, synovial fluid, and synovial tiue) from patients with RA and osteoarthritis (OA) were compared by two-color flow-cytometric analysis. In the $CD4^+$T cell population of peripheral blood, the number of $CD45RO^+$ cells was relatively higher than $CD45RA^+$ cells in both RA and OA patients, but their percentages did not differ from those found in healthy individuals. However, the great majority of $CD4^+$T cells present in synovial fluid and synovial tiue were CD45RO-positive and CD45RA-negative in both patient groups; although $CD4^+$T cells infiltrating both the disease compartments were markedly greater in RA joints, their mean percentages of $CD45RO^+$ cells were not significantly different from those in OA joints. These data indicate that an accumulation of $CD45RO^+$ memory CD4+T cells is a generalized phenomenon during local inflammatory responses in both RA and OA joints, and may be due mainly to the propensity of these cells to preferentially transmigrate into the inflamed joint via adhesion molecules as compared with $CD45RA^+$ naive $CD4^+$T cells.

KEYWORDS: rheumatoid arthritis, ostroarthritis, $CD45RO^+$, $CD4^+$T cells

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Comparative Analysis of CD45RA- and CD45RO-Positive CD4$^+$T Cells in Peripheral Blood, Synovial Fluid, and Synovial Tissue in Patients with Rheumatoid Arthritis and Osteoarthritis

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To determine whether the predominant infiltration with memory CD4$^+$T cells in joints is specific to the local immune and inflammatory response in rheumatoid arthritis (RA), the proportions of CD45RA$^+$ or CD45RO$^+$ cells in the CD4$^+$T cell populations in three different compartments (*i.e.*, peripheral blood, synovial fluid, and synovial tissue) from patients with RA and osteoarthritis (OA) were compared by two-color flow-cytometric analysis. In the CD4$^+$T cell population of peripheral blood, the number of CD45RO$^+$ cells was relatively higher than CD45RA$^+$ cells in both RA and OA patients, but their percentages did not differ from those found in healthy individuals. However, the great majority of CD4$^+$T cells present in synovial fluid and synovial tissue were CD45RO-positive and CD45RA-negative in both patient groups; although CD4$^+$T cells infiltrating both the disease compartments were markedly greater in RA joints, their mean percentages of CD45RO$^+$ cells were not significantly different from those in OA joints. These data indicate that an accumulation of CD45RO$^+$ memory CD4$^+$T cells is a generalized phenomenon during local inflammatory responses in both RA and OA joints, and may be due mainly to the propensity of these cells to preferentially transmigrate into the inflamed joint via adhesion molecules as compared with CD45RA$^+$ naive CD4$^+$T cells.

Key words: rheumatoid arthritis, osteoarthritis, CD45RO$^+$, CD4$^+$T cells

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of unknown etiology. The chronically inflamed synovium is characterized by the presence of large numbers of T cells, particularly those expressing the CD4 antigen characteristic of the helper/inducer phenotype (1-3). These T cells express several activation markers on their surface, such as DR antigens, adhesion molecules, and IL-2 receptors (4). These findings, together with the association of certain major histocompatiblity complex-DR molecules with disease susceptibility (5), indicate that CD4$^+$T cells may have an important role in the pathogenesis of RA.

CD4$^+$T cells as well as CD8$^+$T cells are further divided into two major subpopulations according to the expression of CD45 antigens, a family of isoforms expressed on the cell surface: the high molecular weight form, CD45RA, is predominantly expressed on immunologically virgin T cells, whereas the low molecular form, CD45RO, is expressed on activated or mature memory T cells (6). It is now apparent that differential splicing events of the CD45 gene give rise to these two isoforms (7).

Previous studies on phenotypic characterization of T cells infiltrating in the synovium of patients with RA have shown that the majority are CD4-positive and express CD45RO and CD29 antigens typical of memory cells (8, 9). It, however, remains to be determined whether the predominance of memory CD4$^+$T cells reflects local activation events or is due to their propensity to transmigrate into inflammatory sites. In an attempt to address this question, using two-color flow-cytometric analysis, the proportions of CD45RA or RO antigen expression in the CD4$^+$T cell population present in the peripheral blood, synovial fluid, and synovial tissue from patients with RA were compared with the proportions measured in patients with osteoarthritis (OA), in which synovial inflammation

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is thought to be secondary to cartilage degeneration but not to be primarily mediated by immunological mechanisms (10). We postulated that if the levels of $CD45RO^+$, $CD4^+$T cells reflect mainly the local conversion of $CD45RA^+$ cells to $CD45RO^+$ cells induced by immune and inflammatory reactions, their frequency in RA joints should be greater than that in OA joints.

Materials and Methods

Study patients. Ten patients with RA who met the 1987 revised criteria of the American College of Rheumatism (11), 5 patients with OA, and 5 healthy volunteers were included in this study. Clinical characteristics, laboratory findings, and drug therapies employed in these RA and OA patients are summarized in Tables 1 and 2, respectively. RA patients showed to various degrees the activity of disease as evidenced by elevated erythrocyte sedimentation rate, increased concentrations of serum C-reactive protein, and high titers of rheumatoid factor in the serum, whereas such systemic inflammatory responses were not evident in any of the OA patients.

Cell preparation. Peripheral blood, synovial fluid and synovial tissue samples were simultaneously obtained at the time of total knee replacement for patients with RA and OA. Peripheral blood samples were drawn aseptically from these patients and healthy volunteers into tubes containing Na_2-ethylenediamine-tetraacetic acid.

Table 1 Clinical features of patients with rheumatoid arthritis

	Total study population	Patients from whom synovial samples were obtained
Number of patients	10	7
Male:female	1:9	0:7
Age (years)	55 ± 9.9	57 ± 7.7
Disease duration (years)	11 ± 9.0	13 ± 9.9
Class: I/2/3/4	0/4/6/0	0/3/4/0
Stage: I/II/III/IV	1/0/1/8	0/0/0/7
ESR (mm/h)	57 ± 35	59 ± 43
CRP (mg/dl)	4.6 ± 3.2	4.4 ± 3.8
Rheumatoid factor (IU)	359 ± 412	333 ± 322
Drug treatment		
NSAIDs	10/10	7/7
DMARDs	7/10	5/7

Results are expressed as means ± SEM. ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein; NSAIDs: Nonsteroidal anti-inflammatory drugs; DMARDs: Disease modifying antirheumatic drugs.

Table 2 Clinical features of patients with osteoarthritis

Number of patients	5
Male:female	0:5
Age (years)	72 ± 5.7
Disease duration (years)	11 ± 4.5
ESR (mm/h)	21 ± 10.2
CRP (mg/dl)	0.3 ± 0.6
Rheumatoid factor (IU)	9.1 ± 10.2
Drug treatment	
NSAIDs	10/10

Results are expressed as means ± SEM. ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; NSAIDs: Nonsteroidal anti-inflammatory drugs; DMARDs: Disease modifying antirheumatic drugs.

Synovial fluids were aspirated from the knee joint into heparinized tubes, immediately treated with hyaluronidase (20 U/ml; Mochida, Tokyo, Japan), and filtered over a steel-mesh (pore size 210 μm; Ikemoto, Tokyo, Japan). The cells obtained were washed twice with phosphate buffered saline (PBS). Specimens of synovial tissues from the patients were minced with sterile scissors and incubated for 60 min at 37°C in Hank's balanced salt solution without Ca^{++} and Mg^{++} (Gibco Lab., Life Technologies, Inc., Grand Island, NY, USA) containing 1 μg/ml collagenase (Worthington Biochemical Co., Freehold, NJ, USA) and 40 Kunitz U/ml of deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO, USA). The cell suspensions were filtered over steel-mesh, washed twice with medium, and incubated in a plastic culture dish (Costar Corp., Cambridge, MA, USA) at 37°C in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Gibco Lab.) supplemented with 2 mM L-glutamine, penicillin G, streptomycin, 25 mM Hepes, and 10% heat-inactivated fetal calf serum. The cells that did not adhere to plastic after an overnight incubation were harvested and washed twice with medium. Blood and synovial fluid specimens from the patients were also measured for leukocyte cell count and differentials. Synovial tissues from 3 RA patients and synovial fluid and tissue from an OA patient could not be evaluated for lymphocyte phenotypes since virtually no lymphocytes were obtained. The percentage of lymphocytes in the nonadherent synovial tissue cells from 6 RA patients and 4 OA patients was determined by May-Giemsa staining.

Flow cytometric analysis of lymphocyte subpopulations. The cells prepared were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin

(PE)-conjugated monoclonal antibodies (mAbs) which were all purchased from Becton Dickinson (Mountain View, CA, USA), including FITC-conjugated anti-CD3 (Leu 4; pan-T cells), anti-CD4 (Leu 3a; helper/inducer T cells), and anti-CD45RA (Leu 18; naive T cells) mAbs, and PE-conjugated anti-CD4, anti-CD8 (Leu 2a; suppresser/cytotoxic T cells), anti-CD45RO (Leu 45RO; memory T cells), and anti-CD19 (Leu 18; B cells) mAbs. Each of these mAbs was diluted 5 times with PBS containing NaN_3 (PBS, pH 7.2) and used at the concentration of 0.25 μg/ml. One hundred μl of whole blood samples were incubated in the dark for 60 min at 4 °C in the presence of 20 μl of anti-CD3, anti-CD4, anti-CD8, anti-CD19 mAbs, or combinations of FITC- or PE-anti-CD4 and FITC-anti-CD45RA or PE-anti-CD45RO mAbs. Red blood cells were lysed by 5 min-incubation with 2 ml of FACS lysing solution (Becton Dickinson) followed by washing 3 times with PBS, and the resultant cells were resuspended in PBS. In a similar fashion, 1×10^5 cells of synovial fluid and tissue cells were stained with mAbs and resuspended in PBS. Single or two-color flow cytometric analysis of cells stained with the mAbs was immediately performed on a FACScan flow cytometer (Becton Dickinson) using a 488 nm argon laser, gated for lymphocyte populations. Non-specific stainings were determined using Calibrate beads, leucogate, and control antisera in each analysis. A minimum of 10^4 cells were analyzed for each determination.

Statistical analysis. Results were expressed as the mean ± SEM of the indicated number of individuals. Statistical differences and correlations between two groups were evaluated by the Wilcoxon rank-sum test and the Spearman rank-sum test.

Results

Lymphocytes and CD3⁺, CD4⁺, CD8⁺, and CD19⁺ cell populations in peripheral blood, synovial fluid, and synovial tissue from patients with RA and OA. The number of leukocytes and the percentage of lymphocytes in the total cell population in peripheral blood, synovial fluid, and synovial tissue from RA and OA patients are shown in Table 3. The number of peripheral blood leukocytes tended to be greater in RA patients than in OA patients, although the difference was not statistically significant. Most of the cells infiltrating the cavity of joints from both RA and OA patients were polymorphonuclear cells, and the number of cells counted was markedly greater in RA joints than in OA joints, reflecting the more aggressive nature of synovial inflammation in RA. The percentage of lymphocytes in synovial fluid cells from RA patients varied, however, the mean value did not significantly differ from that of their blood cells; in OA patients, the differential of synovial fluid cells could not be determined due to the lack of cells available for examination. In synovial tissue specimens obtained from RA and OA patients, lymphocytes were found to be the major cell type in the nonadherent cell fraction (72 ± 12 % in 6 RA patients and 64 ± 18 % in 4 OA patients) by May-Giemsa staining.

We next examined the percentages of $CD19^+$, $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes in peripheral blood, synovial fluid, and synovial tissue cells from RA and OA

Table 3 The number of leukocytes, the percentage of lymphocytes, the percentages of $CD19^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells in the lymphocyte population, and CD4/CD8 ratio in peripheral blood, synovial fluid, and synovial tissue from patients with rheumatoid arthritis and osteoarthritis

	Leukocytes (/$\mu\ell$)	Lymphocytes (%)	CD19 (%)	CD3 (%)	CD4 (%)	CD8 (%)	CD4/CD8
Rheumatoid arthritis							
Peripheral blood (n = 10)	6490 ± 591	31.1 ± 4.2	8.5 ± 2.0	60.6 ± 2.4	36.0 ± 3.3	28.2 ± 3.9	1.6 ± 0.3
Synovial fluid (n = 10)	9533 ± 2763	28.5 ± 15.5	4.7 ± 1.8	65.5 ± 4.7	39.1 ± 3.9	35.3 ± 4.6	1.6 ± 0.3
Synovial tissue (n = 7)	ND	ND	9.4 ± 3.8	62.6 ± 8.1	37.8 ± 4.9	31.1 ± 4.7	1.5 ± 0.5
Osteoarthritis							
Peripheral blood (n = 5)	5920 ± 988	35.2 ± 3.7	7.9 ± 2.9	59.1 ± 6.3	35.1 ± 4.7	31.4 ± 3.9	1.0 ± 0.3
Synovial fluid (n = 4)	544 ± 299	ND	1.6 ± 1.0	77.6 ± 5.3	27.0 ± 6.2	47.0 ± 7.5	0.6 ± 0.2
Synovial tissue (n = 4)	ND	ND	4.7 ± 1.4	69.2 ± 14.9	39.6 ± 7.6	32.5 ± 4.5	1.2 ± 0.2

Results are expressed as means ± SEM. ND: Not determined.

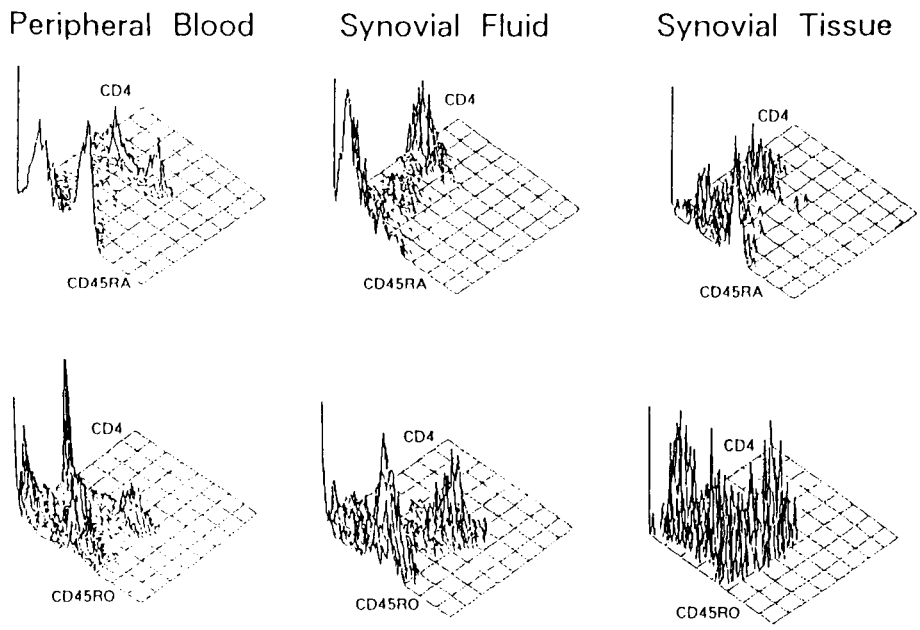

Fig. 1 A representative cytoflurogram of CD45RA- and CD45RO-positive cells in the $CD4^+$T cell populations of peripheral blood, synovial fluid, and synovial tissue in patients with rheumatoid arthritis (RA). Cells were simultaneously obtained from these three samples of RA patients and incubated with a combination of FITC- or PE-conjugated anti-CD4 mAb and FITC-conjugated anti-CD45RA or PE-conjugated anti-CD45RO mAb. The frequencies of CD45RA- and CD45RO-positive cells in the $CD4^+$T cell population were determined by FACScan analysis. In this patient, CD45RA- or CD45RO- positive $CD4^+$T cells were calculated to be 33.9%, 3.3%, 2.8% and 68.4%, 99.6%, 88.4% in peripheral blood, synovial fluid, and synovial tissue, respectively.

patients (Table 3). In synovial fluid and tissue specimens obtained from both patient groups, $CD3^+$T cells were predominant over $CD19^+$B cells; however, the synovial tissue of RA was characterized by higher levels of infiltration with B cells compared with OA tissue. In RA patients, there was no significant difference in $CD4^+$ or $CD8^+$T cells present in the peripheral blood, synovial fluid, or synovial tissue with their mean CD4/CD8 ratios being comparable to the ratio of peripheral blood CD4/CD8 cells from healthy individuals (1.7 ± 0.2). On the other hand, the percentage of $CD8^+$T cells was relatively higher in OA patients; the mean ratios of their peripheral blood, synovial fluid and tissue were lower than the ratio of healthy blood samples ($P < 0.05$).

$CD45RA^+$ and $CD45RO^+$ cells in the $CD4^+$ T cell populations in peripheral blood, synovial fluid, and synovial tissue from patients with RA and OA. We further examined by the two-color immunofluorescence method using FITC- or PE-conjugated mAbs the frequencies of $CD45RA^+$ or $CD45RO^+$T cells in the $CD4^+$T cell populations in peripheral blood, synovial fluid, and synovial tissue from RA and OA patients. Representative profiles of $CD4^+/CD45RA^+$T cells and $CD4^+/CD45RO^+$T cells in these three compartments of a patient with RA are shown in Fig. 1. In peripheral blood $CD4^+$ T cells, the mean percentages of $CD45RA^+$ or $CD45RO^+$ cells in 10 RA (28.6 ± 3.6% and 75.6 ± 3.2%, respectively) and 5 OA patients (28.0 ± 4.2% and 73.0 ± 2.4%) were similar, and were roughly comparable to the levels in 5 healthy individuals (37.8 ± 5.9% and 77.7 ± 4.4%) (Figs. 2 and 3). In contrast, the percentage of $CD45RO^+$, $CD4^+$T cells in the synovial fluid from both RA and OA patients were markedly higher (RA: 97.6 ± 0.8%, $n = 7$; OA: 94.2 ± 3.8%, $n = 4$) and conversely limited numbers of $CD45RA^+$ cells were detected (RA: 4.6 ± 1.3%; OA: 7.7 ± 4.9%). Similarly, most of $CD4^+$T cells present in synovial tissue were positive for the CD45RO antigen in both patients with RA ($CD45RA^+/RO^+$: $6.1 \pm 0.9/90.7 \pm 1.4$%, $n = 7$) and OA ($12.2 \pm 8.0/96.5 \pm 1.3$%, $n = 4$). There was no significant difference between RA and OA patients in terms of the percentages of $CD45RA^+$ or $CD45RO^+$, $CD4^+$T cells in synovial fluid and tissue. In RA patients,

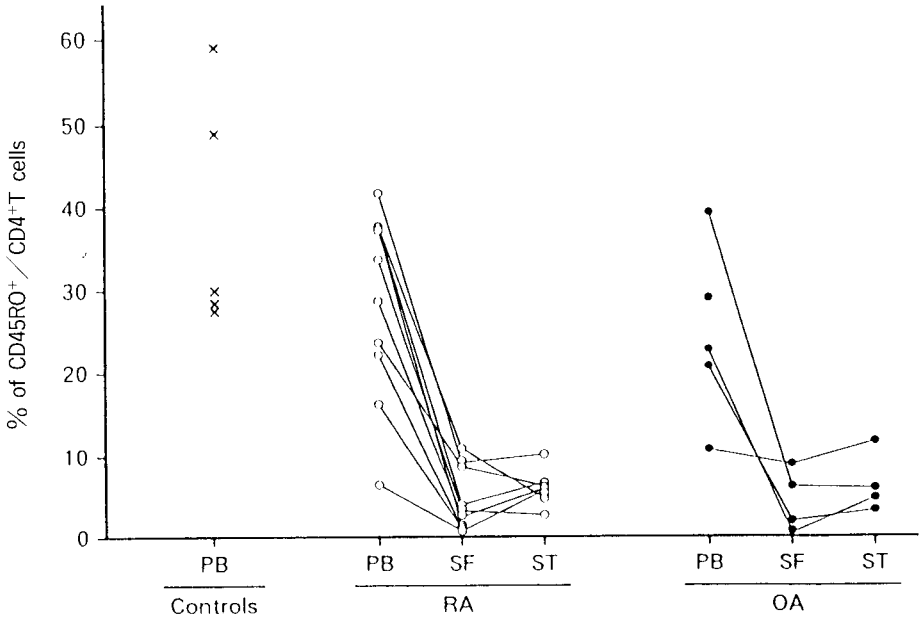

Fig. 2 The percentages of CD45RA-positive cells in the CD4⁺T cell populations in peripheral blood, synovial fluid, and synovial tissue from rheumatoid arthritis (RA) and osteoarthritis (OA) patients. Cells were simultaneously obtained from these three samples of 10 RA patients (○) and 5 OA patients (●) and as a control from the blood of 5 healthy individuals (×). These cells were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD45RA mAbs, and the percentages of CD45RA-positive CD4⁺T cells were determined by two-color flow cytometric analysis as described in the text.

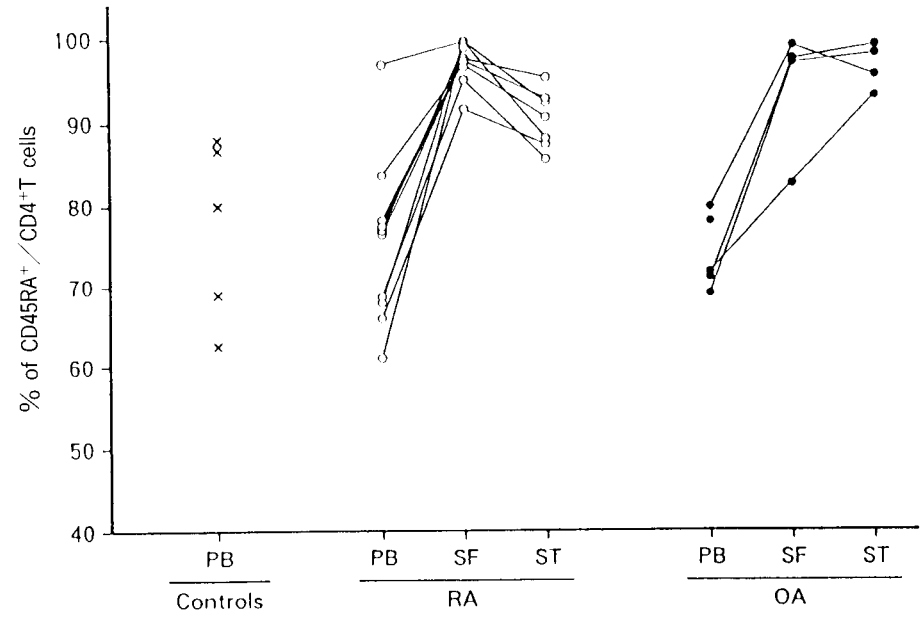

Fig. 3 The percentages of CD45RO-positive cells in the CD4⁺T cell populations in peripheral blood, synovial fluid, and synovial tissue from RA and OA patients. Cells were simultaneously obtained from these three samples of 10 RA patients (○) and 5 OA patients (●) and as a control from the blood of 5 healthy individuals (×). These cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD45RO mAbs, and the percentages of CD45RO-positive CD4⁺T cells were determined by the two-color flow cytometric analysis as described in the text.

neither the percentage of $CD45RO^+$, $CD4^+$T cells in synovial fluid nor that in synovial tissue was found to correlate with indicators of the disease activity such as the values of erythrocyte sedimentation rate and serum C-reactive protein, titers of serum rheumatoid factor, the number of infiltrating leukocytes in synovial effusion, or medication.

Discussion

$CD4^+$T cells are believed to be active participants in the chronic synovial inflammation of RA, although their precise role remains unclear (12). On the other hand, inflammatory reactions in OA joints are induced following the degeneration of cartilage and the role played by T cells in this process is considered to be limited (10). In general, as compared with OA, local inflammation and joint destruction are more aggressive and extensive in RA, often accompanied by systemic inflammatory responses (13). Indeed, the number of $CD4^+$T cells present in both the synovial fluid and tissue samples used in this study was markedly higher in RA patients than in OA patients, indicating that our samples from the two patient groups represent these two forms of arthritis. We found that $CD45RO^+$ cells of the memory phenotype predominated strongly over $CD45RA^+$ cells of the naive phenotype in the $CD4^+$T populations obtained from both the synovial fluid and tissue samples of RA patients when compared with their peripheral blood $CD4^+$T cells, but that the frequency of $CD45RO^+$, $CD4^+$T cells present in inflamed joints of RA was similar to that in OA joints. To our knowledge, this study is the first comparative analysis of phenotypes of $CD4^+$T cells in the three different compartments from the same patients with RA or OA.

A large number of previous studies have shown that the majority of $CD4^+$T cells infiltrating into both the synovial fluid and tissue of RA are mainly of the memory phenotype expressing CD45RO and CD29 (8, 9, 14–18). Some investigators interpreted this as reflecting *in situ* activation events (14, 15) or the preferential transmigration of $CD45RO^+$ cells into inflammatory sites (8, 9, 16–18). *In vitro* studies have shown that $CD45RA^+$T cells differentiate into $CD45RO^+$T cells following activation by antigens and mitogens (19, 20). On the other hand, as compared with $CD45RA^+$ virgin cells, $CD45RO^+$ memory T cells simultaneously express higher densities of adhesion molecules on their cell surface (18, 21). As a result, $CD45RO^+$T cells are thought to be recruited preferentially into inflamed joints where the counterparts of these adhesion molecules are strongly expressed on endothelial cells, macrophages and fibroblast-like cells (22). In fact, $CD29^+$ memory T cells have been shown to be more adhesive than $CD45RA^+$ naive T cells to IL-1-stimulated synovial cells through their expression of lymphocyte function associated antigen-1 (23). However, information regarding CD45RA- and CD45RO-expressing $CD4^+$T cells in the joints, particularly in the synovium, of various arthritides has been so far limited. Previously, marked predominance of helper $CD4^+$T cells with corresponding depletion of naive $CD4^+$T cells in synovial fluid has been demonstrated in various inflammatory arhthritides such as OA, gout, ankylosing spondylitis, psoriatic arthritis, and reactive arthritis, similarly in RA (8, 14, 16, 18), although Emery *et al.* have described the predominance of helper $CD4^+$T cells in synovial fluid as relatively specific to RA (24).

We wished to know whether the accumulation of phenotypically defined memory $CD4^+$T cells seen in RA resulted mainly from *in situ* activation of $CD4^+$T cells or their preferential binding to adhesion molecules expressed at inflammatory sites by comparing the frequencies of $CD45RO^+$, $CD4^+$T cells in the joints of RA and OA patients. If the process of local T cell activation is more important, the predominance of $CD45RO^+$, $CD4^+$T cells would be more evident in RA joints because immunologically mediated inflammation is less prominent in OA. However, the great majority of memory $CD4^+$T cells were similarly observed in both RA and OA joints, despite the quantitative difference in T cell infiltration being significant. In addition, the levels of $CD45RO^+$, $CD4^+$T cells in the RA joint did not correlate with disease activity. These findings favor the notion that most of the memory $CD4^+$T cells in RA joints are cells recruited nonspecifically into the site of the disease via their binding to adhesion molecules and the number of locally activated T cells is very low. Indeed, despite the expression of activation markers such as major histocompatibility antigen-DR, T cell cytokine production and proliferation is extremely restricted in RA joints (25). Therefore, to understand the role of $CD4^+$T cells in the pathogenesis of RA, further investigation at the clonal level of *in vivo*-activated T cells is needed.

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